Translational Renal Genetics

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This dissertation is dedicated to my mother,

Larysa Reznichenko

Paranymphs: Arjan Kwakernaak

Steef Sinkeler

Contents

Chapter 1

General Introduction

Chronic kidney disease

Chronic kidney disease as a global problem

Chronic kidney disease (CKD) is a significant and increasing global challenge for public health. It affects approximately 10% of the general population in industrialized nations, incurring high morbidity and mortality and posing a substantial financial burden to the health care systems. The worldwide rise in the number of patients with CKD and consequent end-stage renal disease (ESRD) necessitating renal replacement therapy (hemodialysis or kidney transplantation) is threatening to reach epidemic proportions.[1-3] As there is a direct relation between the gross national product and the availability of renal replacement therapy,[1,3] the increase in ESRD burden paralleled by the increasing demand for dialysis and kidney transplantation will be most notable in developing countries, since only few countries have robust economies able to meet the challenges posed in terms of health care costs. Therefore, a global and concerted approach to CKD, aimed at finding novel strategies of treatment and prevention, must be urgently adopted in both more and less developed countries to avoid a major catastrophe and reverse the worsening situation.

Potential of genetics in resolving the big issues in chronic kidney disease

CKD is a complex, multifactorial disease with an important genetic component. Together with environmental factors, the genetic predisposition of an individual is crucial for renal disease onset, manifestation and rate of progression. Therefore, identification of the genetic variants involved in susceptibility and progression of CKD, and identification of the mechanism involved in genetically conferred renal risk is critically important. First, it will potentially improve risk prediction and hence allocation of care. Using genetic information, along with classical predictors, will improve identification of individuals at risk for progressive CKD, which will enable targeting interventions and more aggressive primary and secondary prevention to the identified high-risk groups, while preventing the burden of treatment in low risk subjects. Second, it can improve our understanding of biological mechanisms underlying renal function loss by enriching our current knowledge base with novel insights and redefining our prior concepts of kidney pathophysiology. Accordingly, it can identify novel targets for intervention, and hence guide the development of novel tools for diagnosis, prevention and treatment of CKD. Thus, it may also support a basis for improving outcome by personalized therapy in the future.

Kidney function and disease as heritable traits

It had long been noted that there are racial differences in occurrence of kidney disease, with disproportionately higher risk of ESRD in African Americans compared to individuals of European ancestry[4-6]. Further, a population-based analysis revealed that a substantial

proportion of ESRD patients had close relatives with kidney disease. Consistently, familial aggregation studies demonstrated that CKD and ESRD cluster in families.[7-12] All these facts collectively were suggestive of potential genetic background and led to the concept that CKD, probably, involves an inherited, genetic component.

Indeed, heritability studies of kidney function measures have shown a relatively high contribution of genetic factors to the variability of these traits. For instance, heritability of glomerular filtration rate (GFR) was estimated to range from 33 to 75%,[13,14] indicating that between approximately 33 and 75% of the inter-individual variation in GFR could be explained by additive genetic effects. The heritabilities for serum creatinine and calculated creatinine clearance were reported to be 37 and 33-63%, respectively.[15,16] Further, heritability estimates of albuminuria, considered a key sign of kidney damage, range from 16 to 49%.[13,14]

Identifying heritability provided evidence for the importance of genetic factors in determining kidney phenotypes and thus also provided a rationale for searching for common variants associated with renal function and kidney disease.

Large progress has been made over the last decades in the elucidation of the genetic basis of monogenic, Mendelian renal disorders, such as, for instance, autosomal-dominant polycystic kidney disease and the Alport syndrome. More recently, gradual progress is being made regarding CKD in non-diabetic and diabetic patients, as a common, complex disorder.[18-26] In this thesis, emphasis will be on the genetics of CKD as a complex disorder.

Complex trait genetics and methodology

Complex diseases, in contrast to monogenic (Mendelian) disorders, are polygenic and multifactorial, i.e. arise from a combination of multiple genetic and environmental factors, with various environmental exposures acting on genetically susceptible individuals.

It is assumed that complex diseases are caused by common genetic variants, i.e. relatively frequent (>1%) in a given population – a concept known as the “common disease, common variant” hypothesis. These common variants, in contrast to rare Mendelian mutations, have small individual effects with a cumulative biological effect (**Table 1**, **Figure 1**). That is, each common genetic variant individually makes a limited contribution to the phenotypic outcome, and the combined effects of multiple variants in multiple genes, affecting different parts (or pathways) relevant to the complex disorder determine the biological effect that can be either deleterious or protective. Accordingly, the mechanisms involved in the genotype-phenotype relationships are usually complex, and involve multiple interactions, which renders their elucidation quite a challenge.

Table 1. Characteristics of monogenic and complex diseases
(adapted from *Köttgen et al Am J Kidney Dis 2010*)

	Monogenic diseases	Complex diseases
Prevalence	Rare	Common
Public health impact	Small to moderate	Large
Magnitude of associated disease risk	High (often relative risk >5)	Moderate (often relative risk <1.5)
Frequency of genetic risk variants	Rare	Common
Mendelian inheritance pattern	Yes	No
Cause of disease	Single gene mutations, highly penetrant	Multifactorial, multiple genetic and environmental risk factors jointly cause disease

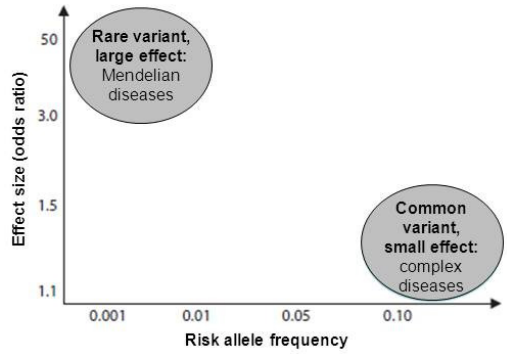


Figure 1. Relationship between effect size and risk allele frequency (adapted from *Böger et al Kidney Blood Press Res 2011*)

Classical genetic approaches: linkage analysis and candidate gene studies

Classical genetic mapping approaches, e.g., linkage analyses, in families with index patients affected by a rare disease proven to be successful in discovering mutations causing rare single-gene diseases with a clear Mendelian mode of inheritance and a clear-cut clinical phenotype. However, the use of these hypothesis-free methods had limited success with complex diseases, reflecting the differences in genetic architecture underlying monogenic and polygenic diseases.

Hypothesis-driven studies, aimed at investigation the association of common genetic variants in biologically plausible candidate genes with phenotypes, represent an additional approach, based on prior biological knowledge implicating target genes with a known or presumed functional role in pathways or diseases. However, since this approach depends on prior knowledge it does not lead to discovery of novel genes. Therefore, in discovery strategies, often a combination of hypothesis-free and hypothesis-testing strategies is applied.

Genome-wide association studies

Recent technological advances led to the availability of affordable microarray-based genotyping platforms, as a methodological basis for whole genome analysis. This resulted in evolvement of a novel powerful instrument in the toolbox of genetic research of complex diseases – genome-wide association studies (GWASs).

GWAS is aimed at determining the statistical relationship between common DNA variants and an observable disease-related characteristics or trait. The most commonly studied variants are single-nucleotide polymorphisms (SNPs), where a single base at a particular genomic location differs among individuals. Contemporary platforms allow parallel genotyping of more than 1 million SNPs. Since 2005, when the first successful GWAS was published, a rapidly expanding number of traits and complex diseases has been studied using this powerful method. By September 2011, a total of 1,596 GWASs for 249 traits was published.[17]

GWAS represent a hypothesis-free type of study design, and is thus unbiased by prior knowledge. The main strength of GWAS is in its potential for discovery of hitherto unsuspected, truly novel genes. Thus, it can guide the discovery of novel pathways of disease, by providing the foundation for novel hypotheses to be tested. Of note, association does not necessarily implicate causality, and functional studies are required to substantiate a pathophysiological role of an associated locus. Moreover, SNPs identified by GWAS are often not involved themselves, but rather implicate corresponding genetic loci. So, GWAS has great discovery potential but requires substantial additional study to translate the impact of discovered loci to disease pathophysiology.

Genetics of chronic kidney disease as a complex disorder

GWASs of renal phenotypes: measures of kidney function and CKD

Renal phenotypes

Proper phenotype definition is critically important for genetic analyses. In nephrogenetic research, a number of different renal traits and diseases have been studied for genome-wide association: serum creatinine, eGFR, albuminuria, CKD, ESRD, etc. Among these, serum creatinine, eGFR and albuminuria are quantitative traits, whereas presence of CKD or ESRD are dichotomous traits. These phenotypes all are assumed to represent CKD one way or another, with ESRD as its most advanced form, and higher creatinine

or albuminuria and lower eGFR allegedly representing earlier forms of renal damage, that may or may not progress to overt CKD.

- The main index of the functional state of the kidney is glomerular filtration rate (GFR) which describes the flow rate of filtered fluid through the kidney. Its direct measurement (e.g. by ^{125}I-iothalamate clearance), although considered the "gold standard", is an invasive and expensive procedure. Owing to the large target sample size dictated by statistical power considerations for a GWAS, it is unfeasible to measure GFR in a population-based study. Therefore, GFR is estimated (eGFR) from serum filtration markers, usually creatinine, and sometimes also cystatin C ($eGFR_{creat}$ or $eGFR_{cys}$, respectively), combined with empirical algorithms that link the serum value of creatinine and/or cystatin to glomerular filtration rate.
- Albuminuria, i.e. elevated urinary albumin, expressed as either as urinary albumin-to-creatinine ratio or as concentration of albumin in urine, is considered to indicate renal damage, irrespective of GFR.
- CKD is conventionally defined in epidemiological studies as eGFR < 60 ml/min/1.73 m^2.
- ESRD is the most severe renal phenotype. As a clinical endpoint of progressive CKD, it is defined as initiation of renal replacement treatment – dialysis or kidney transplantation.

Associated genetic loci

To date, more than 15 GWASs have been performed on various forms of renal phenotype, resulting in discovery of a number of genes[18-26] (**Figure 2**, **Table 2**). A meta-analysis of GWASs of renal function indices in population-based cohorts of approximately 70,000 individuals yielded a total of 28 loci at the genome-wide significant level ($p<5\times10^{-8}$).[19] Collectively, these renal function loci accounted for 1.4% of the variation in $eGFR_{creat}$.

Table 2. Genetic loci associated with phenotypes of renal function

Creatinine and $eGFR_{creat}$[18-20]	$eGFR_{creat}$ and $eGFR_{cys}$[18,19]	CKD[18-20,22]	Albuminuria[21]
GATM-SPATA5L1; CPS1; SLC22A2; TMEM60; WDR37; SLC6A13; WDR72; BCAS3	*UMOD; SHROOM3; LASS2; GCKR; ALMS1; TFDP2; DAB2; SLC34A1; VEGFA; PRKAG2; PIP5K1B; ATXN2; DACH1; UBE2Q2; SLC7A9*	*UMOD; PRKAG2; ANXA9; DAB2; DACH1; STC1*	*CUBN*

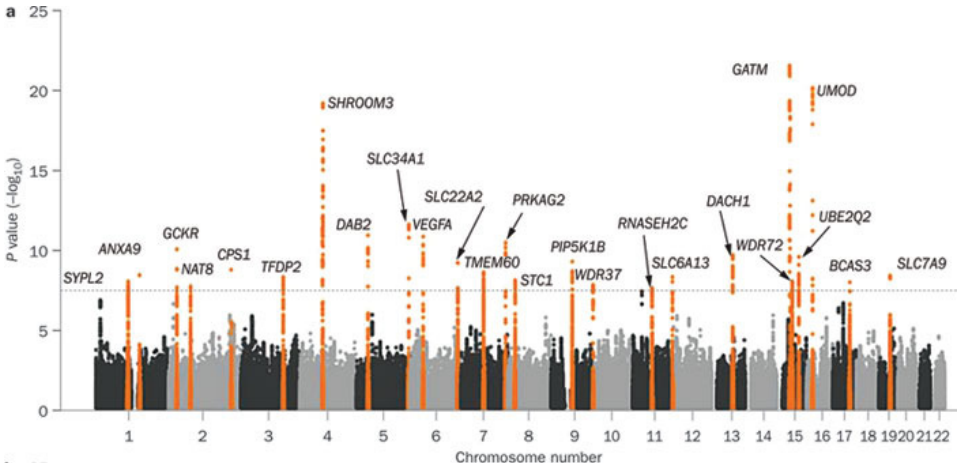


Figure 2. Graphical presentation of the results from a GWAS of eGFR: a Manhattan plot (*Köttgen et al. Nat Genet 2010*).

Chromosomal location is plotted on the x-axis, statistical significance ($-\log_{10}$ p values) for association with eGFR – on the y-axis. The horizontal grey dotted line denotes the threshold of genome-wide statistical significance (5×10^{-8}). The plot displays 25 significant hits.

The majority of the loci associated with lower eGFR showed nominal associations with prevalent CKD.[18,19] In a follow-up study, several of these renal function loci were associated with incident CKD and prevalent ESRD.[22] Other loci for eGFR, however, were not associated with CKD or ESRD: this could implicate that low eGFR is not always an early stage of CKD. Alternatively, there could be selection bias in studies in CKD and ESRD populations, as CKD is associated with increased mortality.

A GWAS of albuminuria traits (urinary albumin-to-creatinine ratio and microalbuminuria) identified one non-synonymous SNP in the *CUBN* gene, explaining 0.2% of total variance of this phenotype.[21] No other loci previously found to be associated with eGFR and CKD showed genome-wide significance for association with albuminuria or vice versa, supporting the proposed concept of distinct, disparate genes responsible for these renal phenotypes.[14,27] Yet, considering the consistent predictive effect of albuminuria for progressive renal function loss, the lack of data on association of *CUBN* variants is somewhat surprising, and warrants better study.

Limitations of GWAS specifically in nephrology research

Despite the large utility of the GWAS approach, there are also some limitations. The requirement for a high degree of statistical significance, along with the fact that risk variants for complex traits usually confer small-to-modest effects, makes a very large sample size essential for adequate statistical power. As a consequence, GWAS has to rely on rather crude phenotypes, as for very large cohorts detailed phenotyping is usually not affordable. The best-studied, crude phenotypes for renal disease, eGFR and albuminuria, are widely available because they are cheap and simple to obtain, but both are only an indirect reflection of the degree of renal damage in an individual, and of the propensity to future progressive renal function loss.

Another important limitation is that most GWASs in nephrology were based on cross-sectional renal data-sets. A main drawback of such designs is that the clinically most relevant phenotypes, namely onset and progression of renal function loss, are not studied. In fact, onset and progression of CKD towards ESRD is a long-term process which typically takes decades, while the rate of renal function loss varies between patients. Accordingly, due to practical and methodological issues, case-control studies are so far the mainstay of GWAS for CKD, but longitudinal studies are definitely warranted to investigate the role of genetic factors in the dynamics of CKD progression.

Finally, as noted above, statistical association does not necessarily implicate causality. Functional studies are needed to investigate whether associated loci are indeed pathophysiologically involved.

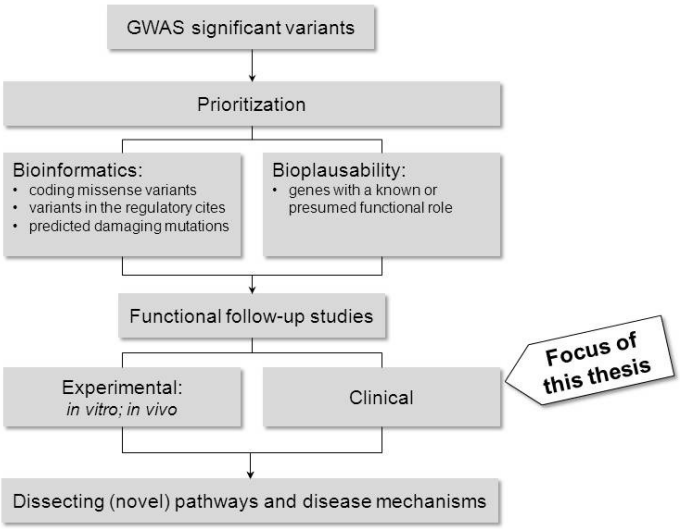
It should be mentioned also, that functional studies are generally time-consuming and costly. Considering the substantial number of candidate loci generated by GWASs, this poses the enormous challenge of prioritizing the loci most likely to be involved in the pathophysiology of renal damage as topics for further study.

From genotype to phenotype in CKD

Prioritization of loci for follow-up

Prioritization of loci from GWAS can be guided by both bio-informatics and by pathophysiological considerations (**Figure 3**), and by their combination. The obvious first-priority candidates for hypothesis-driven post-GWAS follow-up studies are renal loci which are biologically plausible, i.e. those that can be linked to kidney function and/or disease based on existing knowledge on gene expression and its involvement in specific pathways and physiological processes. With regard to the pathophysiological considerations, refining the phenotype can also be useful.

Figure 3. Pipeline of a post-GWAS research to translate genetic findings into pathophysiological mechanisms.



Loci studied in this thesis

In this thesis, in a series of clinical post-GWAS studies, we used several strategies to translate GWAS findings to renal pathophysiology. First, we selected loci with biological plausibility based on prior knowledge of the function of gene in renal (patho-) physiology, namely *SLC22A2,* coding for OCT2, a tubular transporter involved in creatinine secretion; *CUBN*, coding for cubilin, which plays a role in protein reabsorption in proximal tubule, and *UMOD*, coding for uromodulin, the most abundant protein in urine. Second, we studied refined renal phenotypes, allowed by the smaller scale of our studies in a dedicated nephrology setting. Third, we used the unique setting of renal transplantation in a study design allowing both case-control analysis for association of loci with ESRD in native and transplanted kidneys, and dissection of intra- and extra-renal pathways of disease.

SLC22A2

GWASs on renal function so far rely heavily on creatinine-based renal function estimates, i.e. eGFR. Serum creatinine, however, is a complex and composite phenotype and its levels are not only based on the renal phenotype it is assumed to reflect, namely GFR. Creatinine concentration in blood is determined by the interplay of many factors such as its metabolic generation, renal excretion which includes glomerular filtration, tubular secretion and reabsorption, and extra-renal elimination[28-32] (**Figure 4**).

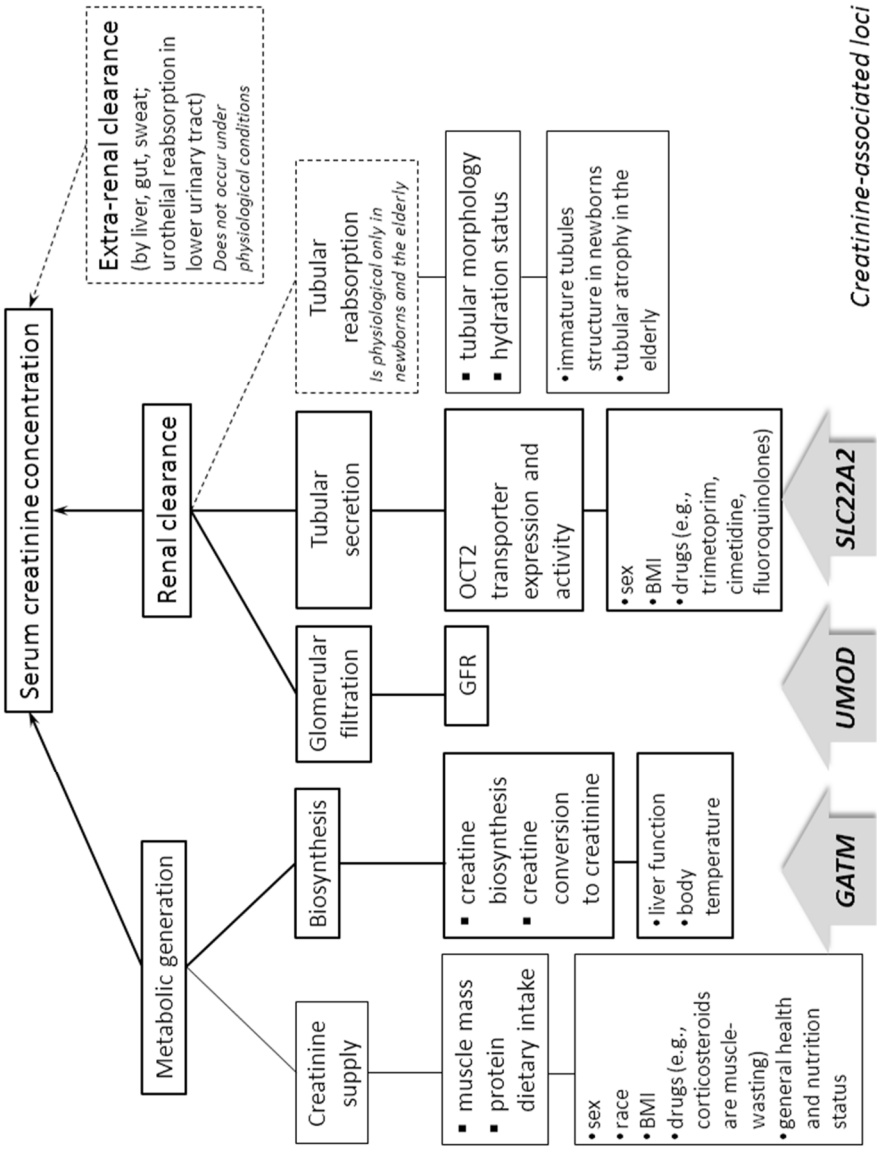


Figure 4. Schematic overview of determinants of serum creatinine levels and the presumed functional involvement of three examples of creatinine-associated genetic loci.

Therefore, assuming that serum creatinine phenotype specifically reflects GFR might lead to erroneous conclusions. Dissecting the exact biological mechanism underlying the genetic association with serum creatinine can guide the interpretation of GWAS findings and help to translate them into a clinically meaningful message. It is, thus, important to separate genetic loci that affect concentrations of creatinine independently of underlying renal disease from those that truly reflect disease-association (i.e. decline of renal function in terms of GFR). Indeed, among the loci associated with creatinine and/or eGFR was *GATM*, a gene involved in creatinine metabolism, and *SLC22A2*, that may affect tubular creatinine handling.

Using a second measure of renal function, e.g. cystatin C-based eGFR, helps to distinguish between true renal function loci and creatinine production/secretion loci. Applying this approach, the previously reported renal loci were classified into two groups: the loci that were associated with both $eGFR_{creat}$ and $eGFR_{cys}$, and, thus, are likely to be involved in renal function and susceptibility to CKD, and the loci that were associated with $eGFR_{creat}$ only and, therefore, were suspected to affect creatinine production and secretion.[13,19,33]

SLC22A2 was considered as a likely creatinine secretion locus, which makes it stand apart from the other, supposedly GFR- or creatinine biosynthesis-associated, genes identified by GWAS of renal function traits. In the abovementioned GWA studies the *SLC22A2* SNPs were associated with GFR estimated from serum creatinine but not from cystatin C.[19,33] The *SLC22A2* gene encodes the organic cation transporter 2, OCT2. From its general biology it is known to be a predominant transporter involved in the renal secretion of creatinine. In the kidney it is expressed in the basolateral membrane of proximal tubule cells, where it mediates tubular uptake of creatinine from the peritubular capillaries, as an initial step in its vectorial transport to the apical cell membrane followed by secretion into the lumen. The active tubular secretion of creatinine is assumed to be relatively insignificant (it accounts for an additional 10-20% of urinary creatinine excretion), and therefore is neglected in the routine estimation of renal function in clinical practice. However, existence of tubular secretion of creatinine as an additional mechanism of its clearance translates into systematic error (bias) in creatinine-based GFR estimation equations.

The significance of the abovementioned facts is two-fold. First, it points to the complexity of creatinine metabolism and importance of proper interpretation of the results of GWAS of creatinine-related phenotypes. Second, it suggests that the genetic variation in the *SLC22A2* locus may have consequences for the accuracy of creatinine-based GFR estimation methods.

CUBN

Cubilin has long since been studied in renal disease. In the kidney, cubilin is expressed predominantly in the apical brush border of proximal tubular cells, where it plays a key role in the receptor-mediated endocytotic reabsorption of albumin and other low-molecular-weight proteins. The essential role of the cubilin-megalin complex in the reuptake of albumin by the proximal tubule has been demonstrated in animal experimental studies.[34-36] Consistently, a rare autosomal-recessive disorder caused by mutations in the *CUBN* gene which encodes cubilin, Imerslund-Gräsbeck disease, (OMIM #261100, Finnish type) typically manifests with varying degrees of proteinuria as a result of a molecular defect leading to inefficient proximal tubular protein reabsorption[37] (**Figure 5**).

Renewed interest in cubilin was fueled by a recent GWAS of albuminuria[21] that found an association with the *CUBN* locus. As albuminuria is a main predictor of progressive renal damage, it would be plausible to investigate whether common genetic variation in *CUBN* associates with CKD. Alternatively, the association between albuminuria and the *CUBN* locus might reflect urinary albumin loss due to selective alteration of albumin uptake in the proximal tubulus.

UMOD

Among renal loci, a prominent place is taken by *UMOD,* which has been reproducibly identified in multiple cohorts as one of the top loci associated with renal function parameters. Also, Mendelian mutations in *UMOD* have been described, such as glomerulocystic kidney disease, familial juvenile hyperuricemic nephropathy and medullary cystic kidney disease 2.

The *UMOD* gene encodes uromodulin (or Tamm-Horsfall protein), which is expressed exclusively in the kidney in a peculiar pattern – it localizes in the thick ascending limb of the loop of Henle specifically omitting the *macula densa*[38-40] (**Figure 6**). Uromodulin functions, among other, as a protective molecule against urinary tract infections and stone formation. Nevertheless, its exact physiological role, despite more than 60-years of investigation,[41] remains obscure.[38-40]

Uromodulin is excreted into urine and is in fact long since known as the most abundant tubular urinary protein in healthy subjects. Its constitutive physiological excretion occurs from the 15th week of gestation on,[42] and it is such an inherent urinary constituent that it is used for forensic identification of urine.[43,44] This feature is unique among the currently identified loci for renal function, as it constitutes a non-invasive intermediate phenotype that could be highly useful to unravel genotype-phenotype relationships.

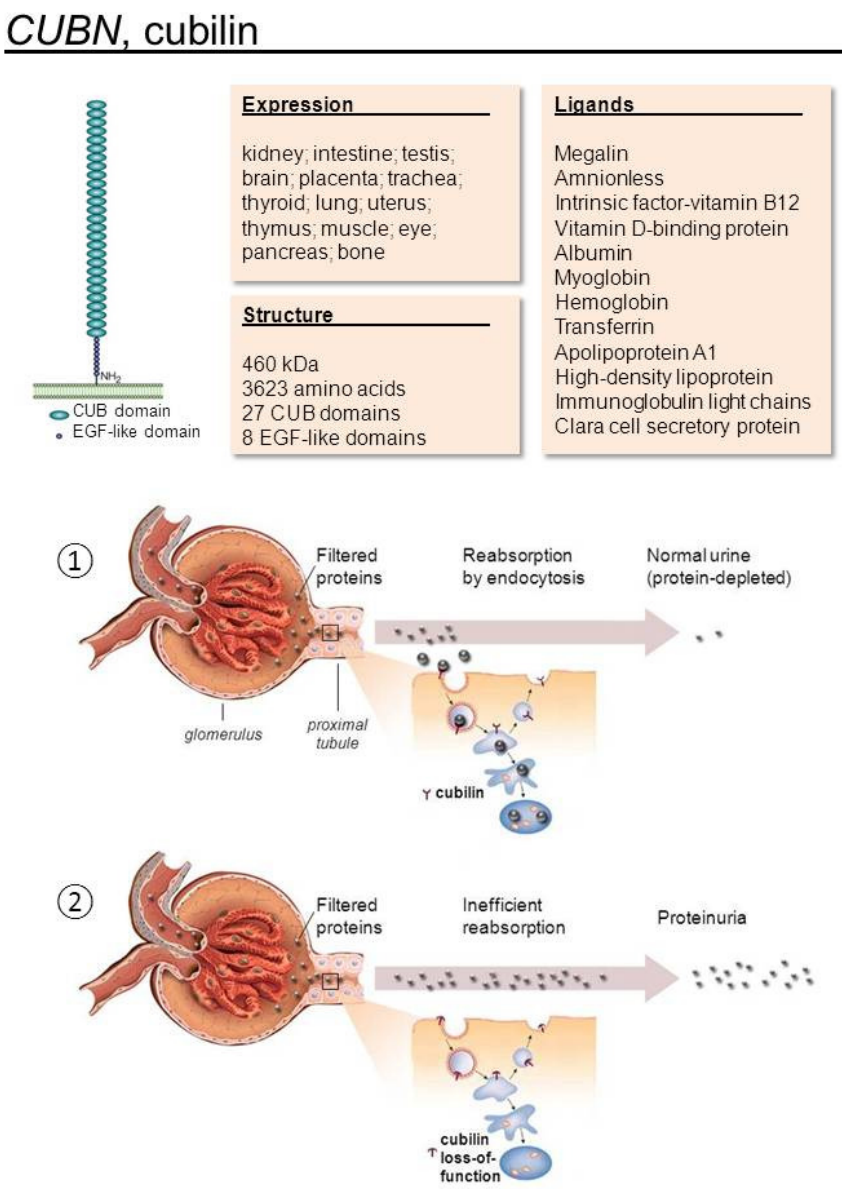


Figure 5. Cubilin structure and function.

Cubilin is a multiligand endocytic receptor. ① Low-molecular-weight proteins, e.g. albumin, are filtered into the primary urine and, under normal conditions, virtually completely reabsorbed by proximal tubule cells via the cubilin-mediated endocytosis pathway. ② Functional cubilin deficiency results in defective tubular re-uptake of filtered proteins clinically manifesting as low-grade tubular proteinuria.

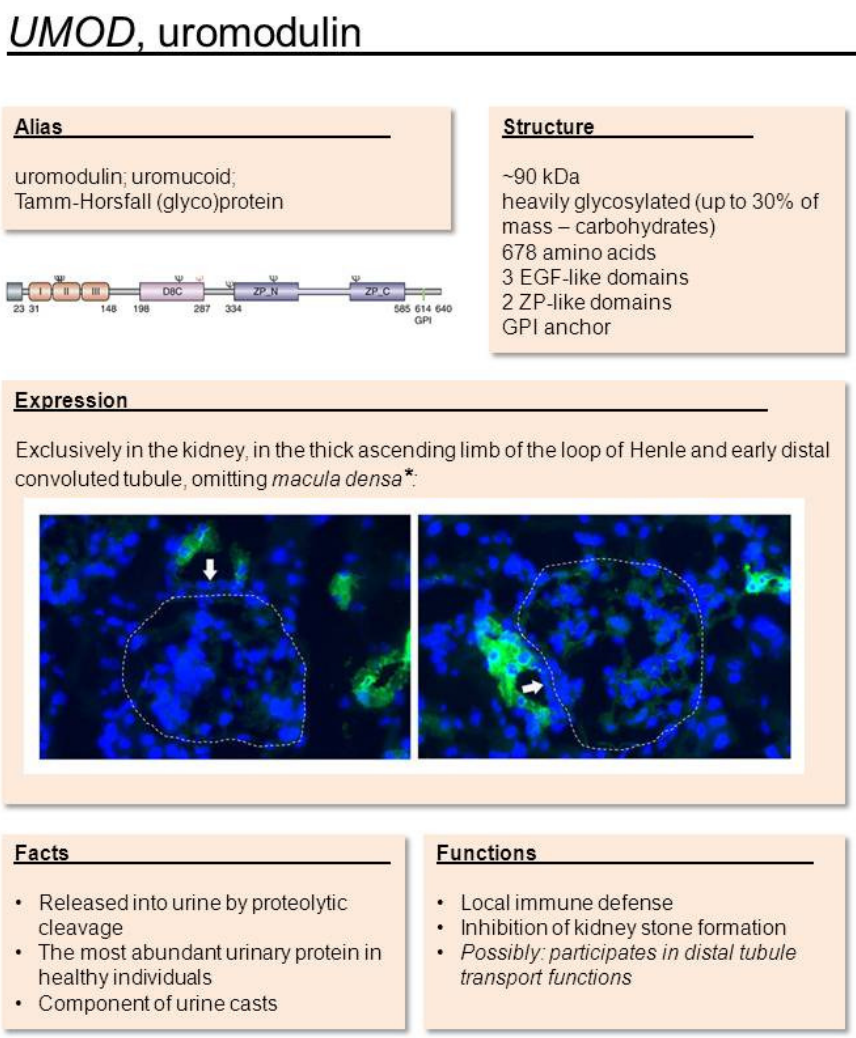


Figure 6. Uromodulin structure and function.
*Own data: Uromodulin immunofluorescence (green) on acetone-fixed rat renal cryosections using sheep polyclonal anti-uromucoid antibody and rabbit-anti-sheep/FITC conjugated secondary antibody (Abcam Inc., Cambridge, MA); nuclei (blue) stained with DAPI. Glomeruli cross-sections are encircled with white dashed lines. The *macula densa* areas are indicated with arrows and are negatively stained demonstrating absence of uromodulin protein expression.

Recent data suggest a role of genetic variation in the *UMOD* gene, uromodulin excretion and the susceptibility to renal damage. Several studies highlighted a region upstream from the *UMOD* gene containing a linkage disequilibrium block of several SNPs (e.g., rs12917707, rs4293393 and rs13333226) which were repeatedly shown to be associated with uromodulin urinary concentration.[45-47] Furthermore, genetic variation in the *UMOD* gene was associated with hypertension,[47] and, moreover, genetically determined elevated urinary uromodulin levels were associated with increased risk of incident CKD.[45] This is of particular interest, as it might implicate that urinary uromodulin could be an intermediate phenotype for the genetic susceptibility to renal damage.

However, physiological regulation of urinary uromodulin excretion is rather complex and, probably, goes beyond the straightforward single locus genetic effect. Besides, uromodulin is subject to diverse environmental influences. Therefore, the genotype-phenotype associations between variation in the *UMOD* gene, urinary uromodulin excretion and the susceptibility to renal damage warrants better in-depth investigation.

Kidney transplantation as a dissecting tool for nephrogenetic research

GWAS data on renal traits so far are limited to subjects with their native kidneys, in the general population and in renal disease populations. Studies in renal transplant recipients would be of great interest as well, for several reasons. First, renal transplantation recipients represent a population with a high burden of disease. Identification of genes associated with loss or preservation of the transplanted kidney might be highly useful for risk stratification as well as contribute to identification of new treatment targets. Second, the renal transplantation setting has particular characteristics that might be relevant to obtain insights in the genetic basis of renal damage in native kidneys as well:

- Genotypes of recipients and donors can be contrasted in a case-control design, with recipients being cases (patients with ESRD requiring kidney transplantation) and donors serving as controls (individuals either without kidney disease (deceased donors) or specifically selected for superior kidney function to be eligible for donation (living donors)). Using kidney donors, instead of the general population, as controls may augment statistical power to reveal the subtle genetic effects expected from common variants, thus representing an “extreme” case-control design.

- A transplant population provides the opportunity to study renal function loss in both native and transplanted kidneys through investigation of ESRD before and graft failure (GF) after transplantation. Availability of long-term post-transplant follow-up for GF in the recipients enables implementation of a longitudinal study design, in a dedicated setting, without loss to follow-up. Importantly,

reproducing case-control genotype-phenotype associations in the longitudinal study serves as a (proof-of-principle) independent replication of the results between the native and transplanted kidney, within the same individual.

- Finally, transplantation represents a peculiar and unique setting from a genetic point of view: an organ with its own genotype functions in an organism with another genotype. The elegant study design involving testing of both donor and recipient genotype for association with phenotypes allows to discriminate between local (intra-renal) and systemic (extra-renal) processes influencing renal traits.

Outline of this thesis

The general aim of this thesis is to extend data from recent GWAS on renal traits towards the clinically relevant renal phenotypes.

We aimed to fill the gap between associated genetic loci and disease phenotypes by studying relevant intermediate phenotypes, and exploring additional, previously uninvestigated kidney phenotypes.

We followed up the previous GWASs focusing on the most prominent, biologically plausible renal loci, namely: *UMOD*, *CUBN* and *SLC22A2* (**Figure 7**).

Chapters 2 to 4 of this thesis are devoted to *UMOD*. In **chapter 2** we evaluate the predictive performance of urinary uromodulin excretion for incidence of graft failure in renal transplant recipients. In **chapter 3** we investigate non-genetic determinants of urinary uromodulin in healthy subjects as well as in renal patients. Questions of intra-day and day-to-day variability of uromodulin excretion are addressed in part 1 of chapter 3. In part 2 we study the effects of dietary sodium intake and renoprotective pharmacological interventions on urinary uromodulin levels in CKD patients. **Chapter 4** investigates the association of the *UMOD* gene polymorphism with end-stage renal disease in native kidneys and graft failure in transplanted kidneys, as well as with urinary uromodulin in transplanted kidneys.

In **chapter 5** we investigate the *CUBN* genetic variants for association with phenotypes of renal damage (proteinuria) and progressive function loss (end-stage renal disease and graft failure, respectively) in a kidney transplant cohort.

In **chapter 6** we attempt to dissect the previously reported association of genetic variation in *SLC22A2* with estimated GFR in the glomerular and the tubular component of creatinine clearance. We hypothesize that the association is mediated by tubular creatinine handling rather than by glomerular filtration. Accordingly, we test whether the

genetic variation in *SLC22A2* affects the accuracy of estimating true GFR from serum creatinine, by modulating the tubular component of renal creatinine excretion.

Finally (**chapter 7**), blood urea level is often used as a generic measure for the metabolic impact of renal function impairment. As a first step to elucidate the genetic basis of blood urea level as an additional and potentially relevant renal trait, we performed a GWAS for blood urea, a novel renal phenotype for which associated genetic loci have not been identified in Caucasians thus far.

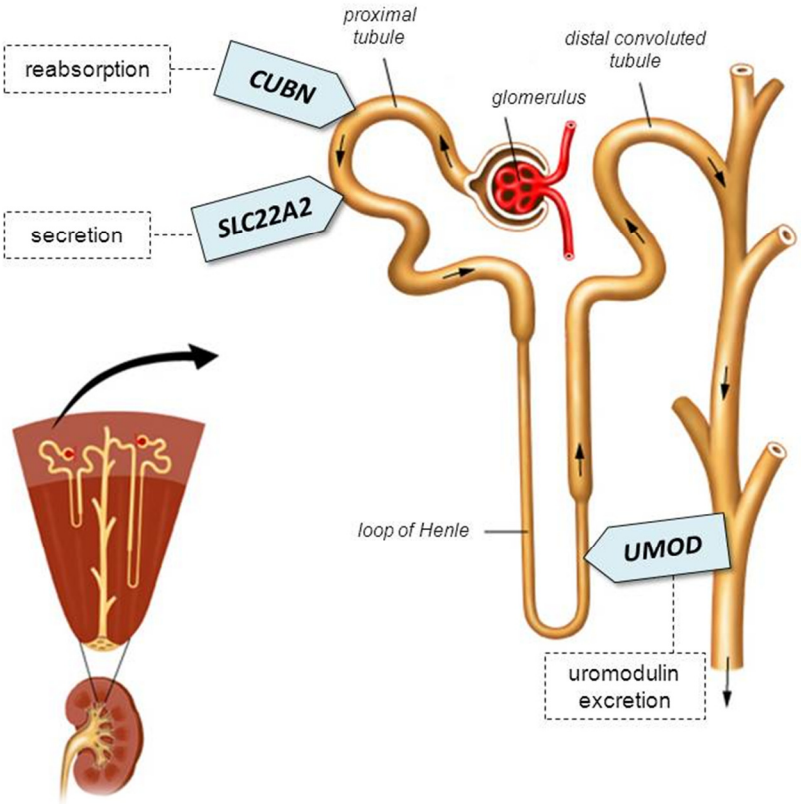


Figure 7. The genetic loci studied in this thesis: relative spatial localization of expression in the kidney coupled with a relevant physiological process.

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Chapter 2

Urinary uromodulin is elevated in renal transplant recipients and associated with graft failure in a bimodal fashion

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Abstract

Urinary uromodulin predicts renal prognosis in native kidneys, but data are conflicting. We investigated its prognostic impact for graft failure (GF) in renal transplant recipients (RTR, n=600).

Uromodulin concentration was measured cross-sectionally in RTR at 6.0 [2.6-11.4] years post-transplant, in matched patients with native chronic kidney disease (CKD) and healthy subjects.

During a follow-up of 5.3 [4.5-5.7] years GF had occurred in 7% of RTR. Median uromodulin excretion (mg/24h) was 20.4 in RTR, 11.6 in CKD and 5.7 in controls (p<0.001). There was a curvilinear association between uromodulin excretion and baseline renal function (p<0.003) and death-censored GF, with 5.5%, 11.5% and 4.0% cases in subsequent uromodulin excretion tertiles, respectively (p=0.002). On multivariate Cox regression analysis hazard ratios for GF for the 1st and 3rd tertiles were 0.37 (p=0.01) and 0.21 (p=0.001) respectively. In 59 RTR allograft biopsies were reviewed. Interstitial fibrosis and tubular atrophy were more severe in the middle tertile (p=0.007).

We conclude that urinary uromodulin is elevated in RTR and associated with graft function, morphology and outcome in a bimodal fashion. Dissection of the disparate mechanisms of GF prediction by urinary uromodulin might provide new clues for its alleged pathogenetic significance in progressive renal function loss.

Abbreviations:

CKD, Chronic Kidney Disease; ELISA, Enzyme-linked Immunosorbent Assay; GF, Graft Failure; IFTA, Interstitial Fibrosis and Tubular Atrophy; RTR, Renal Transplant Recipients.

Introduction

Uromodulin, or Tamm-Horsfall glycoprotein, is the most abundant protein in the urine of healthy subjects. It is exclusively expressed in the cells of the thick ascending limb of Henle's loop and the early distal convoluted tubule and excreted in the urine by shedding. Uromodulin functions, among other, as a protective molecule against urinary tract infections and stone formation, and a number of other roles and mechanisms has been proposed.[1-3]

Urinary uromodulin has been widely studied for its potential as a renal biomarker. It was found to be reduced in several renal conditions, i.e diabetic nephropathy, polycystic kidney disease, tubulointerstitial nephropathy,[4] acute tubular necrosis,[5] nephrolithiasis,[6] medullary cystic kidney disease-2 and familial juvenile hyperuricemic nephropathy,[7] and in miscellaneous conditions, such as preeclampsia,[8] hyperprostaglandin E-syndrome[9] and hypothyroidism.[10] Elevated uromodulin was reported in the hyperfiltration stage of diabetes mellitus type 1,[11] Balkan endemic nephropathy,[12] after uninephrectomy due to kidney donation[13] and in pregnancy.[8]

Interestingly, urinary uromodulin was reported to have prognostic value for the subsequent course of renal function, albeit not uniformly. Decreased uromodulin concentrations predict development of renal failure within 20 years in diabetes mellitus type I[14] and onset of renal insufficiency immediately after liver transplantation.[15] In contrast, recent data showed elevated urinary uromodulin levels to associate with progressive renal function decline and increased risk for CKD in the general population.[16] Few data are available on uromodulin excretion in renal transplantation,[5,13,17-19] and data on its prognostic value in renal transplantation are lacking altogether. In the present study, therefore, we investigated urinary uromodulin levels in a large single center cohort of renal transplant recipients (RTR) and assessed its predictive value for development of late graft failure (GF).

Methods & Materials

Study design and patients

The details of the current RTR cohort have been published previously.[20] Briefly, a total of 606 outpatient RTR (age 51.5±12.1 years; 55% male; at 6.0 [2.6-11.4] years post-transplant) with a functioning graft >1 year were enrolled and followed up during 5.3 [4.5-5.7] years for graft failure, which was censored for death and defined as return to dialysis or re-transplantation. At the study enrollment 24h urine samples were collected, processed by centrifugation (1000×g for 10 min), aliquoted and stored at -80°C until uromodulin measurement. The Institutional Review Board approved the study protocol (METc 01/039).

Uromodulin measurement

All urine samples were pretreated by low-speed centrifugation to pellet large particulate matter (cellular debris, etc.) and, in the same time, avoid loss of large uromodulin aggregates, if any.[21] Uromodulin urinary concentration was measured by commercially available enzyme-linked immunosorbent assay (ELISA) kit (MD Bioproducts, St. Paul, MN, Tamm-Horsfall Glycoprotein ELISA, catalog number M036020) according to the manufacturer's instructions. The principle of the assay is based on a colorimetric sandwich immunoassay utilizing a polyclonal antibody against human uromodulin as the capture antibody and a biotinylated polyclonal antibody against human uromodulin as the detection antibody. For this assay, the interassay coefficient of variation (CV) is 10.5% at a mean concentration of 21.8 ng/mL and 12.2% at 95.1 ng/mL; the sensitivity is less than 0.75 ng/mL.

Prior to measurements of the RTR samples we run pilot experiments to determine optimal assay conditions. Performance of the ELISA has been tested and optimal urine samples dilution has been defined (>1:200). Results obtained after samples pretreatments with previously published TEA buffer[22] or 7M urea were not different from using supplied with the kit buffer only (pH=7.33). Also, we obtained reference values for uromodulin levels in healthy subjects. In these subjects (n=18; 9 men, 9 women, median age 25 yrs, range 19-42 yrs) uromodulin concentration was 3.8 [2.7-5.1] µg/ml with a day-to-day CV of 35% during 5 consecutive days and its excretion was 5.7 [2.8-10.3] mg/24h.

Immediately after the ELISA procedure absorbance reading was performed at 450 nm. A standard curve was generated with each set of samples by reducing the data using 4-parameter logistic curve fit. Uromodulin concentration was calculated from the standard curve. Uromodulin concentration values which were below the detection limit (in n=10 samples) were set to the lowest value in the dataset. The data are presented as uromodulin 24h excretion (mg/24h) as well as uromodulin concentration – absolute (µg/ml) and indexed to urinary creatinine (ng/mmol), to allow for comparisons with different data formats in other publications. Uromodulin levels were also measured in native CKD patients (n=20, matched for age, sex and renal function with the RTR population).

Biopsy scoring

In the transplant cohort, we selected a subset of patients (n=59) for whom episode renal allograft biopsies were available within a time-window of 22 months before till 14 months after urine sampling at the study enrollment (=uromodulin measurement) and reviewed the archival slides of biopsies sections stained by standard methods for routine microscopy (hematoxyline and eosin-, Jones- and PAS-staining). Selection criteria were minimal possible time interval between biopsy taking and uromodulin measurement and sufficient amount of allograft tissue material for histological diagnosis assessed by

glomeruli number and section area. Histological changes were scored according to the Banff '97 classification revised in 2007.[23] In addition, interstitial fibrosis and tubular atrophy (IFTA) and hyaline casts were assessed semi-quantitatively. The biopsies assessment was performed by a pathologist, which was blinded to uromodulin data and clinical information.

Statistical analyses

Analyses were performed with SPSS 16.0 (SPSS Inc., Chicago, IL). Normally distributed variables are presented as means±SD, non-normally distributed ones – as medians [25th-75th percentile] unless other is indicated. Urinary uromodulin concentrations were measured in a total of 606 transplant recipients; after exclusion of cases with missing data as a result of dataset quality control, statistical analyses were performed on a final sample of n=600. The subjects were divided in tertiles based on uromodulin excretion level. Differences between tertiles were tested by one-way ANOVA in case of a parametric variable or the Kruskal-Wallis test in case of a non-parametric variable; the chi-square test was used in case of a categorical variable. Kaplan-Meier survival analysis was performed for analysis of graft loss. A Cox regression analysis was used to test predictive performance of uromodulin excretion.

Results

Uromodulin levels are elevated in renal transplant recipients and associated with graft failure in a bimodal fashion

In baseline 24-h urine, uromodulin concentration ranged from 0.3 µg/ml to 96.9 µg/ml with a median of 8.9 µg/ml; urinary uromodulin/creatinine ratio was 1.8 [1.1-3.0] ng/mmol. Uromodulin 24h excretion was 20.5 [13.2-35.3] mg/24h, which was substantially elevated comparing to CKD patients and healthy controls (**Figure 1**).

The most important transplantation-related and outcome parameters are presented as break-up by tertiles of uromodulin excretion (**Table 1***)*. Duration of pre-transplant dialysis, cold and warm ischemia times, proteinuria, study enrollment time-point and duration of follow-up beyond the baseline did not differ between the tertiles. Also there were no differences between uromodulin excretion tertiles by sex, age (both recipients' and donors'), smoking habits, body size parameters (BMI and BSA), blood pressure, diabetes and glucose homeostasis parameters, blood lipid profile, anti-hypertensive, immunosuppressive and diuretic medications, blood uric acid and history of gout, sodium excretion (data not shown). Renal function as estimated by creatinine clearance at the study enrollment was the lowest in the 2nd uromodulin tertile. Serum urea was elevated in the 2nd tertile.

Table 1. Baseline transplant-related and outcome parameters subdivided by tertiles of uromodulin excretion.

	Tertiles of uromodulin excretion, mg/24h			*p*-value
	10.2 [5.7-13.2]	**20.4 [17.6-24.1]**	**45.9 [35.3-62.0]**	
N	200	200	200	
UMOD concentration, µg/ml	4.7 [3.0-6.6]	8.7 [7.2-10.7]	17.2 [12.3-23.8]	<0.001
UMOD/uCrea, ng/mmol	0.86 [0.48-1.18]	1.78 [1.49-2.25]	3.78 [2.87-5.83]	<0.001
Recipient age, yr	51±13	50±12	52±11	NS
Recipient sex: male, n (%)	117 (58,5)	106 (53,0)	107 (53,5)	NS
Donor age, yr	37±15	37±16	37±16	NS
Cold ischemia time, h	22 [14-26]	21 [13-28]	22 [15-27]	NS
Total warm ischemia time, min	35 [30-43]	35 [30-46]	37 [30-45]	NS
Time post-transplant at enrollment, yr	5.6 [2.5-11.4]	6.1 [2.7-11.1]	6.1 [2.7-12.0]	NS
Follow-up beyond enrollment, yr	5.2 [4.5-5.7]	5.2 [4.5-5.7]	5.3 [4.5-5.7]	NS
Baseline proteinuria, g/24h	0.2 [0.1-0.5]	0.2 [0.0-0.5]	0.2 [0.0-0.5]	NS
Baseline serum creatinine, µmol/l	147±56	154±60	142±61	0.005
Baseline creatinine clearance, ml/min	61±23	59±22	64±22	0.003
Baseline serum urea, mmol/l	9.4 [7.0-13.5]	10.3 [7.9-13.7]	8.9 [6.8-11.9]	0.007
Death-censored GF, n (%)	11 (5.5%)	23 (11.5%)	8 (4.0%)	0.008

UMOD, uromodulin; uCrea, urinary creatinine; GF, graft failure

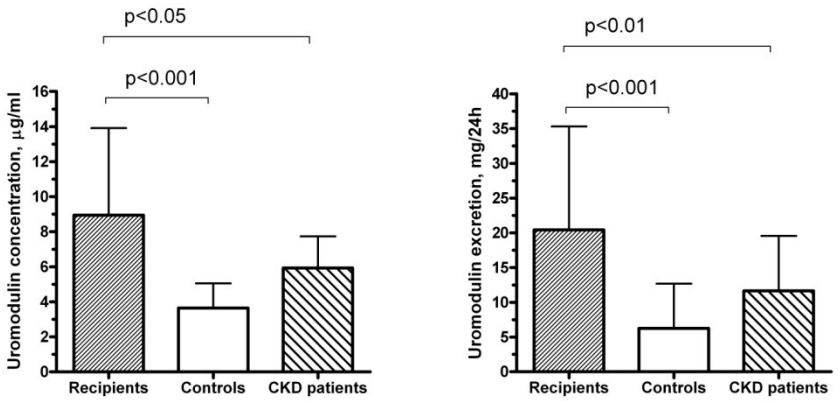

Figure 1. Uromodulin urinary concentration and 24h excretion in renal transplant recipients (n=600) versus healthy controls (n=18) and chronic kidney disease (CKD) patients (n=20). Columns represent respective medians of values, error bars – interquartile range. P-values are given to indicate statistical significance of observed differences between the groups.

During the follow-up of 5.3 [4.5-5.7] yrs, 42 (7.0%) RTR developed GF. According to the tertiles statistics, occurrence of GF was significantly increased in the middle tertile of uromodulin excretion: 5.5%, 11.5% and 4.0% of cases were observed in the 1st, 2nd and 3rd tertiles respectively (p=0.008). Consistently, Kaplan-Meier survival analysis revealed worse graft survival for the middle tertile of uromodulin excretion as compared to the 1st and 3rd tertiles (**Figure 2**).

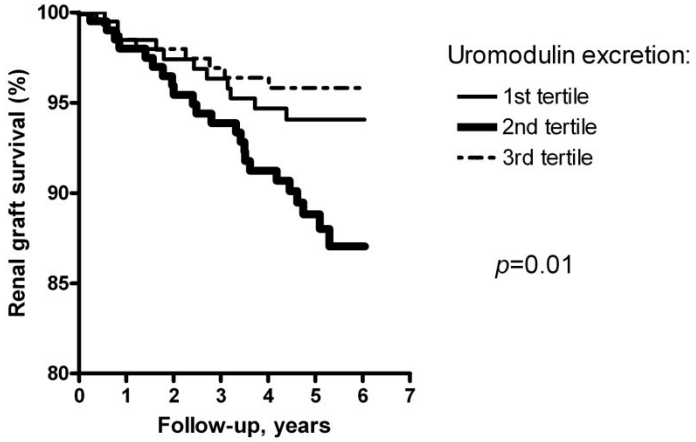

Figure 2. Curves of renal graft survival by tertiles of uromodulin excretion. The logrank test showed significance of the differences between the respective curves (p=0.01).

Since both 1st and 3rd tertiles displayed unidirectional trends, for subsequent regression analyses the middle tertile was used as a reference category. In a multivariate Cox regression analysis hazard ratios for graft failure for the 1st and 3rd tertiles of uromodulin excretion were 0.37 [95% CI 0.17-0.79] (p=0.01) and 0.21 [0.08-0.50] (p=0.001) respectively in the model adjusted for baseline serum creatinine and proteinuria (**Table 2**). Adjustment for donor age did not substantially influence regression coefficients and p-values (data not shown).

Table 2. Uni- and multivariate Cox regression analyses of graft failure predictors.

Tertiles of uromodulin excretion	Crude (univariate) model		Model, adjusted for serum creatinine		Model, adjusted for serum creatinine and proteinuria	
	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
1st	0.48 (0.24-0.99)	0.047	0.32 (0.15-0.68)	0.003	0.37 (0.17-0.80)	0.011
2nd	1.00	-	1.00	-	1.00	-
3rd	0.35 (0.15-0.77)	0.009	0.21 (0.09-0.50)	<0.001	0.21 (0.08-0.50)	0.001

HR, hazard ratio; 95% CI, 95% confidence interval

Uromodulin excretion is associated with allograft interstitial fibrosis and tubular atrophy

Allograft biopsies were analyzed in 59 patients out of the RTR cohort. In this sample, mean age was 48.79±12.11 yrs (63% males), follow-up beyond baseline was 4.7 [2.5-5.4] yrs, during which 15 patients (25.4%) developed graft loss. Uromodulin excretion was 20.9 [14.0-30.1] mg/24h, its urinary concentration – 8.4 [6.0-12.4] µg/ml. Likewise in the total cohort, subjects were divided into tertiles of uromodulin excretion (**Table 3**).

Table 3. Allograft histology parameters subdivided by uromodulin tertiles.

	Tertiles of uromodulin excretion			***p*-value**
	13.6 [8.8-14.7]	**20.7 [18.7-22.3]**	**43.1 [28.5-57.3]**	
N	19	20	20	
Uromodulin concentration, µg/ml	5.7 [4.1-6.9]	8.2 [7.2-9.4]	17.8 [10.9-22.2]	<0.001
Interval biopsy-urine collection, months	-10.6±1.7	-7.9±2.1	-9.0±2.1	NS
Interstitial fibrosis score (Banff, ci)	0.28±0.14	0.95±0.26	0.32±0.15	0.015
Tubular atrophy score (Banff, ct)	0.28±0.14	1.11±0.23	0.32±0.15	0.001
IFTA semi-quantitatively, mean score	0.31±0.09	0.96±0.24	0.33±0.12	0.007
Tubular atrophy: yes, n (%)	4 (21.1%)	13 (65.0%)	6 (30.0%)	0.012
Tubular atrophy: moderate-to-severe, n (%)	1 (5.3%)	9 (45.0%)	3 (15.0%)	0.008
Baseline creatinine clearance, ml/min	63 [43-80]	49 [39-68]	51 [36-64]	NS
Death-censored graft failure, n (%)	4 (21%)	7 (35%)	4 (20%)	NS

Most of the biopsies preceded baseline urine collection; duration of the interval was not different between the tertiles. uromodulin excretion was associated with Banff scores of tubular atrophy and interstitial fibrosis (IFTA): these lesions were significantly more prevalent in the middle tertile of uromodulin excretion. Semi-quantitative assessment of IFTA confirmed these findings (p=0.007) contributing more precision and higher resolution. If analyzed as a categorical variable, tubular atrophy, especially severe, was mostly observed in the middle uromodulin tertile, whereas normal, non-atrophic tubuli were present at both lower and higher uromodulin levels (1st and 3rd tertiles, respectively) [**Table 3**]. Hyaline casts quantity was not associated with uromodulin level (data not shown).

Discussion

Urinary uromodulin was substantially elevated in RTR as compared to native CKD patients and healthy controls. Uromodulin excretion was associated with subsequent late graft failure in a non-linear, bimodal fashion with a lower risk in subjects with the lowest and highest uromodulin excretion. This bimodal association was also found between uromodulin excretion and baseline renal function, and, in a subgroup, allograft histology with less IFTA in subjects with low and high uromodulin levels.

To our knowledge this is the first report of elevated urinary uromodulin in RTR. The differences between RTR, CKD patients and healthy subjects were similar for uromodulin concentration and total 24-hour excretion, thus excluding bias by differences in urinary volume and supporting the robustness of the finding. The elevated uromodulin excretion in RTR is remarkable, considering that RTR have only one functioning kidney. Increased excretion of uromodulin could be due to its increased synthesis in the kidney, increased shedding into urine, or both.[2,3] Unfortunately, our data do not allow to distinguish between these possibilities. Factors known to affect uromodulin excretion are sodium status,[24,25] genetic factors[16,26,27] and renal damage.[28,29] In our study sodium status did not explain the differences between RTR and CKD (data not shown). In our population genetic data were not available, and an association with the severity of renal damage was indeed present – albeit bimodal. However, it is remarkable that average uromodulin excretion was elevated as compared to native CKD with an approximately similar renal function. Assay factors should be considered, as urinary uromodulin from RTR has a different glycosylation pattern than urinary uromodulin in native CKD.[18] We assume that our ELISA was robust against this since the antibodies recognize peptide epitopes of uromodulin and not its carbohydrate part. Uromodulin is known for its potent aggregation and polymerization capacity, which can be an obstacle for its precise measurement.[2,21,22,30] In our study, we used commercial ELISA, which we extensively tested in pilot experiments prior to main measurements, and ensured adequate handling of the samples which were assayed at high dilution in a buffer with alkaline pH. All these methodological measures were directed to provide the highest solubilization of uromodulin to ensure its most accurate quantification.[21,22]

We tested the predictive performance of uromodulin excretion for subsequent GF and found a bimodal association, which is relatively unusual for biomarkers. The bimodal association was also present for baseline renal function, with a slightly, but significantly lower renal function in the middle tertile. Adjusting for the difference in renal function on a Cox regression analyses, however, did not annihilate the predictive value of urinary uromodulin for GF. Data in the literature on the prognostic impact of uromodulin are contradictory so far. *Decreased* uromodulin concentrations predicted development of renal failure in diabetes mellitus type 1[14] and onset of renal insufficiency after liver transplantation,[15] whereas other studies showed that *elevated* uromodulin levels associate with development of CKD in the general population.[16] The discrepancies between the above studies and our current data might well be due to differences in the

nature extent of renal damage in the different populations and in differences in modifying factors. Our data, however, suggest the possibility of a different explanation for the discrepancies, namely a bimodal association, that can mimic a positive or a negative association, as well as the lack of an association when the study population is relatively small or contains only a limited range of renal function or uromodulin excretion. Our study provides the largest population so far with data on the prognostic impact of uromodulin, the number of subjects in other studies ranging between 34[15] and 200,[16,23] usually in case-control design. Thus, it may have been the larger power and the prospective cohort design that allowed to identify the bimodal association pattern.

In our study we confirmed the bimodal pattern also for the association between uromodulin and renal morphology. In a subset of patients in whom a renal biopsy was available within a reasonable time frame from the uromodulin measurement, we found more severe IFTA in the middle tertile of uromodulin excretion. This further enhances the robustness of our findings, but of course the interpretation of the morphological data is limited as the biopsies represent a distinct selection, with biopsies being taken on clinical indication, at points in time that were many months apart from the urine sampling for uromodulin measurement.

Literature data reports an association between uromodulin and renal function (serum creatinine and urea, creatinine clearance, GFR)[4,16,27,29] and morphological damage.[28,29] In our study, we also found these parameters to be associated with uromodulin levels, although in a bimodal fashion.

Our interpretation of the findings is based on assumption of uromodulin inductional changes. Uromodulin is a pleiotropic and overall “protective” protein, therefore, it seems plausible to expect its compensatory up-regulation upon renal damage to execute its protective role. Thus, we hypothesize that in case of renal damage it is up-regulated and contributes to reparation processes. However, in diseased condition with *persistent* renal injury, initial compensatory up-regulation is followed by a state when uromodulin excretion is fixed to some intermediate level due to failure of further up-regulation (for instance, because of tubular cells atrophy). If that is the case, group of lower values of uromodulin excretion (1st tertile) would represent cases without renal damage and favourable outcome; higher values (3rd tertile) – cases of successful uromodulin up-regulation and, consequently, also favorable outcome; average values (2nd tertile) – failure to up-regulate uromodulin (or exhaustion of up-regulation mechanism due to long-lasting damage triggers) and progressive decline in renal function up to graft loss.

In agreement with this hypothesis, we observed low values of uromodulin excretion, comparable with the 1st tertile, in healthy subjects (p=0.1), while biopsies analyses revealed morphologically preserved, non-atrophic tubuli in case of both low and high uromodulin excretion values. Thus, it seems tempting to conclude that low UMOD levels are physiological norm and intact tubuli morphology is a prerequisite of ability to increase uromodulin production, as we speculate, in response to damage triggers.

Longitudinal data on uromodulin excretion over time, in association with development of renal damage, would be useful to substantiate this hypothesis.

A limitation to our study is the lack of certainty on uromodulin origin. We assume that urinary uromodulin originated from the allograft, but a contribution of native kidneys is not completely ruled out for all patients. However, native urine production seems rather unlikely at a median of 6 years post-transplant.

Conclusion

We found an excretion of uromodulin to be associated with renal allograft function, morphology and outcome in a bimodal fashion. In our RTR population, both lower and higher uromodulin levels consistently associate with higher creatinine clearance, lower IFTA and reduced risk of graft failure. We believe that these intriguing findings will encourage further research and finally dissection of disparate mechanisms of graft loss prediction by uromodulin will provide new clues for its role in progressive renal disease.

Acknowledgements

We thank Wendy Dam for the excellent technical assistance.

Supplementary Material

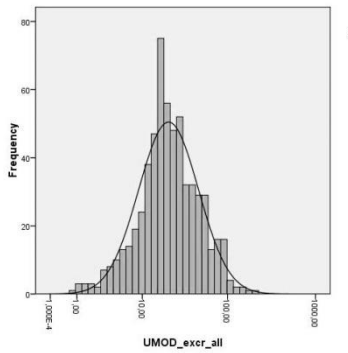

Figure S1. Histogram of uromodulin excretion values distribution (n=600).

X axis is log10-transformed. The distribution approximates normality.

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Chapter 3

Environmental determinants of urinary uromodulin

Part 1

Uromodulin urinary excretion: a time-course study in healthy volunteers

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Introduction

Uromodulin, or Tamm-Horsfall glycoprotein, is long known as the most abundant urinary protein in healthy subjects. It is encoded by the *UMOD* gene which in humans is expressed in the cells of the thick ascending limb of Henle's loop. Uromodulin is thus produced exclusively in the kidney, is excreted into urine from the 15th week of gestation on,[1] and is such an inherent urinary constituent that it is used for forensic identification of urine.[2,3]

Uromodulin functions, among other, as a protective molecule against urinary tract infections and stone formation, although its exact physiological role, despite more than 60-year-history of investigation, remains obscure.[4-7]

Due to potential biomarker properties, uromodulin has been extensively studied in a wide range of renal and extra-renal diseases and in a number of pathological conditions its urinary excretion was found to be changed: either reduced (in diabetic nephropathy, polycystic kidney disease, tubulointerstitial nephropathy[8], acute tubular necrosis,[9] nephrolithiasis,[10] *UMOD* mutations,[11] preeclampsia,[12] hyperprostaglandin E-syndrome,[13] hypothyroidism[14]) or elevated (in hyperfiltration stage of diabetes mellitus type I[15], Balkan endemic nephropathy,[16] post-nephrectomy due to kidney donation[17] and in pregnancy[12]).

Moreover, uromodulin have been tested for prognostic utility. A number of studies attempted to predict prospective disease outcomes by preceding level of uromodulin urinary concentration. For instance, it has been shown that decreased uromodulin concentrations predict development of renal failure in diabetes mellitus type 1[18] and onset of renal insufficiency after liver transplantation.[19] Another study showed elevated urinary uromodulin levels to associate with progressive renal function decline and increased risk for CKD,[20] and we have previously observed a bimodal association between uromodulin excretion and incidence of renal graft failure.[21]

The abovementioned prognostic studies employed a prospective longitudinal design with a single baseline uromodulin measurement and subsequent follow-up of the patients. Importantly, literature data contain warnings about allegedly high day-to-day variability in uromodulin excretion, up to radical statements of inappropriateness of studies based on a single uromodulin measurement.[22,23] However, data on uromodulin variability grounded on sufficient number of observations over substantial period of time are lacking.

Furthermore, a diurnal rhythm is present for urinary volume as well as for many urinary compounds (e.g., albumin, β_2-microglobulin, IgG, transferrin, sodium).[24-27] Whether excretion of uromodulin is subject to diurnal fluctuations is unknown.

To address these issues, we studied uromodulin urinary levels over 5 consecutive 24h periods ("days"), each consisting of 4 sub-periods ("portions"), in 20 young healthy

volunteers. Our research question was whether uromodulin levels in the urine indeed undergo significant changes from day to day (infradian variability) or in the course of the day (diurnal variability). To test that, we studied a time-course of three outcomes of interest: uromodulin urinary concentration, uromodulin excretion and urine volume, in relation to time-related predictors: “day” and “portion”. Since the inter-individual variability might be attributable to between-subject factors, we took into account such parameters as age, sex and body mass index.

Methods & Materials

Study design

Definition of health in our study included: no history of renal disease, no medication intake, normal dipstick test, normal renal function by 24-h creatinine clearance. As uromodulin is produced by renal tubules, we also checked urinary levels of kidney tubular markers (NGAL, L-FABP and H-FABP) to confirm intact tubular morphology in the participants.

Thus, 20 healthy subjects (11 men and 9 women, age range 19-42) were recruited from the hospital personnel and asked to collect 24-h urine during 5 consecutive days, with 4 timed collections (portions) per day: 1 – first morning void; 2 – all the subsequent voids until 17:00 hr; 3 – all the subsequent voids after 17:00 hr on until the following portion; 4 – last void before night sleep and voids during night if any. We did not aim to investigate equal times periods but representative parts of the day. Layout of our study design in shown in **Figure 1**.

Each portion was collected separately into an individual sterile graduated 500 mL plastic container without additives. Participants were instructed to keep the containers in the refrigerator and return them the next day after collection. Volumes of the portions were recorded. Subsequently, specimens of the individual portions were centrifuged at low speed to remove large particulate matter (1000×g at 4 °C for 10 min), aliquoted in fresh polypropylene tubes and stored at -80°C until assayed.

Although in a well-motivated and properly instructed population, composed of health care and research professionals, a proportion of incomplete urine collections is presumed to be small, we assessed completeness of the 24-h urine collections by comparing measured urinary creatinine and the one estimated from the equation: (22 - (age / 9)) × kg in women and (28 - (age / 6)) × kg in men.

Once during the study (on day 3 to 5 of collection) a non-fasting venous blood sample was obtained by venipuncture and used for creatinine measurement. Creatinine concentration was also assessed in the corresponding 24-h urine collection, and

creatinine clearance was calculated as U×V/P, where U - creatinine concentration in 24-h urine, V - 24-h urinary volume, and P - creatinine concentration in plasma.

Participants were allowed to maintain their usual occupational and leisure activities and liberal diet during the study, avoiding heavy physical exercise and alcohol consumption. Data on age, weight, height, medication/food supplement intake were obtained by a self-reporting uniform questionnaire. Body mass index (BMI, kg/m^2) was calculated as weight, kg / $(height, m)^2$.

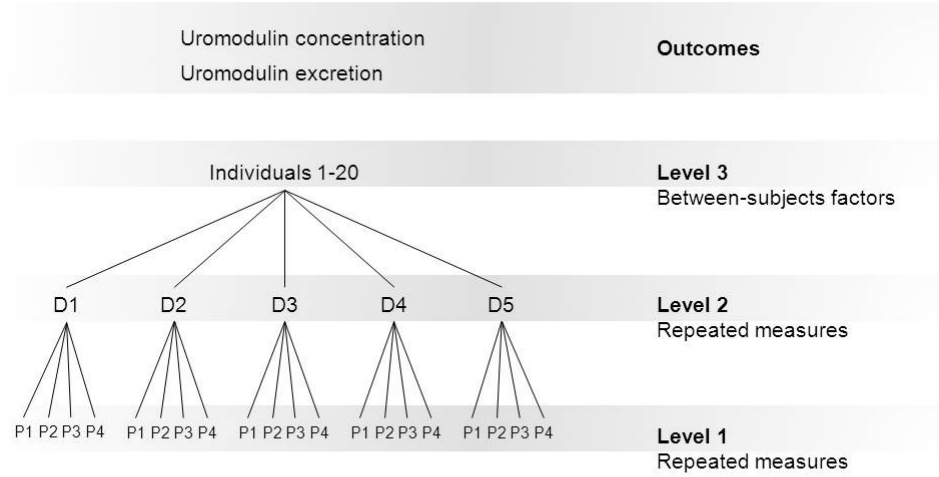

Figure 1. Hierarchy of the multilevel data for our study design. D1-5 designate corresponding days, P1-4 – urine portions.

Uromodulin measurement

Uromodulin urinary concentration was measured by a commercially available enzyme-linked immunosorbent assay (ELISA) kit (MD Bioproducts, St. Paul, MN) according to the manufacturer's instructions. Briefly, the principle of the assay is based on a colorimetric sandwich immunoassay utilizing a polyclonal antibody against uromodulin bound on the surface of microwells as the capture antibody and a biotinylated polyclonal antibody against uromodulin as the detection antibody. According to the manufacturer, the interassay coefficient of variation (CV) is 10.5% at a mean concentration of 21.8 ng/mL and the intra-assay CV is 9.2% at a mean concentration of 22.9 ng/mL; the sensitivity is less than 0.75 ng/mL.

Prior to performing the assay, urine samples were thawed, brought to room temperature, mixed by vortexing avoiding foaming and diluted 1:200 (as determined to be optimal in pilot experiments) with the dilution buffer (pH=7.33) supplied with the assay kit. Immediately after the ELISA procedure absorbance reading was performed at 450 nm. With each set of samples a standard curve, which included seven two-fold dilutions of the supplied with the assay standard protein (150 ng/mL), was generated. Uromodulin concentration was calculated from the standard curve by reducing the data using a 4-parameter logistic curve fit regression function.

The data are presented as absolute uromodulin concentration (ng/mL) and uromodulin excretion – mg/portion, calculated as uromodulin concentration in a urinary portion × portion volume, and mg/24h, calculated as a sum of portions during the corresponding day. In case of absence of a timed urine collection (portion) due to reported absence of urination over the respective time period, urine volume and uromodulin concentration were set to zero for the corresponding portion (15 out of maximally possible 400 collections, 3.75%). Uromodulin concentration values which were below the detection limit (in 17 out of 385 analyzed urine collections, 4.4%) were set to the lowest value in the corresponding assay.

Urinary biomarkers measurements

NGAL (neutrophil gelatinase-associated lipocalin), H-FABP (fatty acid binding protein, heart type) and L-FABP (fatty acid binding protein, liver type) urinary concentrations were measured with commercial ELISA kits (HycultBiotech, Uden, the Netherlands, catalog numbers HK330, HK402 and HK404 for NGAL, H-FABP and L-FABP, respectively), according to the manufacturer's instructions.

Statistical analysis

An analysis of covariance model was fitted to the different uromodulin responses – its urinary concentration (ng/mL) and excretion (mg/portion or mg/24h, calculated as uromodulin concentration × urine volume). The factors subject, day, and portion and all two-way interaction terms were included in this model and considered random. The corresponding variance components are denoted by σ_S^2, σ_D^2, σ_P^2, σ_{SD}^2, σ_{SP}^2, and σ_{DP}^2, respectively. The residual variance component, which is confounded with the three-way interaction term is denoted by σ_E^2. The random effects are corrected for the covariates sex, age, and BMI. The model parameters are estimated with restricted maximum likelihood.

The diurnal and infradian variability of the uromodulin responses for an individual urine sample are defined by the intra-day variability ($\gamma^2_{\text{Diurnal}} = \sigma^2_P + \sigma^2_{SP} + \sigma^2_{DP} + \sigma^2_E$) and the inter-day variability ($\gamma^2_{\text{Infradian}} = \sigma^2_D + \sigma^2_{SD} + \sigma^2_P + \sigma^2_{SP} + \sigma^2_{DP} + \sigma^2_E$), respectively. From these measures of variability intraclass correlation coefficients (ICCs) can be determined for an average response of different numbers of samples taken within and between days. The ICCs are defined by

$$ICC_{\text{Diurnal}} = 1 - \frac{\gamma^2_{\text{Diurnal}} / (DP)}{\sigma^2_S + \left(\gamma^2_{\text{Infradian}} - \gamma^2_{\text{Diurnal}}\right)/D + \gamma^2_{\text{Diurnal}} / (DP)}$$

$$ICC_{\text{Infradian}} = 1 - \frac{\gamma^2_{\text{Infradian}} / D + \gamma^2_{\text{Diurnal}} / (DP)}{\sigma^2_S + \left(\gamma^2_{\text{Infradian}} - \gamma^2_{\text{Diurnal}}\right)/D + \gamma^2_{\text{Diurnal}} / (DP)},$$

with *P* the number of portions taken on one day and *D* the number of days for collecting samples. It should be noted that the ICC_{Diurnal} is always equal or larger than the $ICC_{\text{Infradian}}$. The ICCs can be interpreted as the amount of information that is provided by the number of urine samples. The higher the ICC's the more precise the uromodulin responses have been determined for a subject. The consequences of a low or high ICC for taking one sample on just one day is indicated in the following table:

		ICC_{diurnal}	
		Low	High
$ICC_{\text{Infradian}}$	Low	Multiple samples on one day (*P*>1, 24-h collection would be advisable) from different days are needed (*D*>1)	Multiple samples from different days are needed (*D*>1)
	High	NA	One sample is satisfactory (*P*=*D*=1)

The measures of variability and the ICCs will be estimated to quantify the amount of changes uromodulin measurements undergo.

Results

In all included individuals urinary levels of NGAL, L-FABP and H-FABP were either undetectable or below the reference values, thus confirming healthy status of the kidney. The measured/expected ratios for urinary creatinine were between 0.7 and 1.1 confirming accuracy of the collections. Renal function by 24-h creatinine clearance was within the normal range. Over the course of 5 days, median 24-h uromodulin concentration and uromodulin excretion were 3.47 µg/mL and 6.25 mg/24h, respectively (**Table 1**). In **Figure 2** summarized data (averaged for 20 subjects) on uromodulin concentration, excretion and urinary volume, observed intra-day (**A**) and inter-day (**B**), are visualized. Dynamics of the individual values observed from day to day is presented in **Figure 3**.

Table 1. Study participants characteristics

Age, years	26.5 (24-30.5)
Sex: males / females, n	11 / 9
BMI, kg/m^2	22 (21-24.8)
Serum creatinine, µmol/L	82.0 (67.5-87.8)
24-h creatinine clearance, mL/min	138 (122-165)
Uromodulin concentration, µg/mL	3.47 (2.18-4.44)
Uromodulin excretion, mg/24h	6.25 (2.32-11.87)
Urine volume, L/24h	1.6 (1.2-2.2)

Data are presented as medians (IQR)

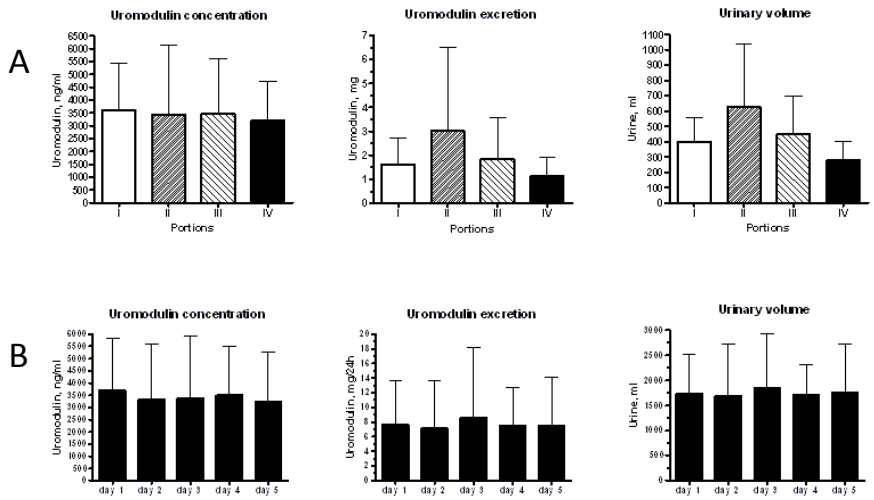

Figure 2. Intra-day (A) and day-to-day (B) values of, respectively, uromodulin concentration, uromodulin excretion and urine volume, observed in 20 healthy subjects. The data are presented as medians and IQR.

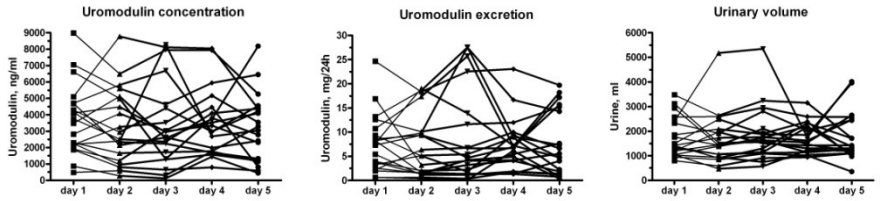

Figure 3. Dynamics of uromodulin concentration, uromodulin excretion and urine volume observed over five consecutive 24-h periods. Each individual line represents a study subject.

Variance estimates of "day", "portion" and of the corresponding interaction term approximated zero for uromodulin concentration (**Table 2**). This is indicative of the absence of systematic bias and suggests no physiological rhythmic fluctuations (diurnal or infradian) for uromodulin urinary concentration. However, for uromodulin excretion effect of "portion" was higher than zero (although non-significant), most probably, reflecting interference by urinary volume diurnal pattern.

Both diurnal and infradian ICCs for uromodulin concentration and excretion, measured in random urine portion (one timed collection on one day) were low and similar in magnitude (**Table 3**). Expanding the number of observations by assessing uromodulin in urine portions taken on several days increased the ICCs proportionately. Using 24-h urine collection for uromodulin measurement already gave moderate-to-high ICCs, which reached high values when collection over several days was employed.

Table 2. Results of a linear mixed model on the dependent variables: uromodulin concentration and uromodulin excretion.

Outcomes	Estimates of:								
	Random effects of within-subject factors and interactions						Fixed effects of between-subject factors		
	Day	Portion	Day* Portion	Subject	Subject* Day	Subject* Portion	Age	Sex (male)	BMI
Uromodulin concentration, ng/mL	0	0	0	**3.06×10^{6}**	1.18×10^{5}	0.43×10^{5}	55.61	-635.39	-205.31
Uromodulin excretion, mg	0	0.49	0	1.56	0.14	**2.02**	0.08	0.07	-0.16

Statistical significance in shown in **bold**.

Table 3. Intraclass correlation coefficients (ICCs) for diurnal and infradian variability of uromodulin concentration and excretion.

Number of portions	Number of days	Uromodulin outcome			
		Concentration		Excretion	
		$ICC_{Diurnal}$	$ICC_{Infradian}$	$ICC_{Diurnal}$	$ICC_{Infradian}$
1	1	33.5%	32.3%	22.1%	20.3%
	2	49.8%	48.9%	35.2%	33.7%
	5	71.0%	70.5%	57.0%	56.0%
4*	1	66.9%	64.4%	53.1%	48.8%
	2	79.9%	78.4%	68.5%	65.6%
	5	90.7%	90.0%	84.1%	82.7%

*sum of 4 portions of the day = 24-h urine collection

Discussion

Reference values for urinary uromodulin reported in the literature vary dramatically, which can be most probably explained by using different laboratory techniques for its assessment. However, our primary interest was not the absolute values but relative, temporal changes in uromodulin excretion occurring in a given individual from day to day and in the course of the day under normal conditions.

A number of previous reports pointed to existence of a large variation in daily uromodulin excretion.[5,6,22,23] Studies attempting evaluation of uromodulin excretion on consecutive days showed that values varied considerably both within and between subjects, which led to the conclusion that studies based on a single baseline uromodulin measurement may not detect any significant results due to the large variability.

However, the above mentioned studies were based on rather low numbers of observations over a short period of time (e.g. two healthy male volunteers during four consecutive days[23]). Besides, the studies employed different designs and approaches (e.g. assessing uromodulin concentration in 24-h urine collections on consecutive days[23] vs 2-h interval collections over 24 h[22]).

We studied uromodulin urinary levels over 5 consecutive 24h periods, each consisting of 4 intervals (“portions”), in 20 healthy volunteers to address problems of both day-to-day and intra-day variability of uromodulin. We revealed no obvious diurnal or infradian rhythms for uromodulin concentration in the urine. However, uromodulin excretion, calculated as a product of uromodulin concentration and urinary volume, reflected the diurnal pattern of the latter.

The potential practical implications of our study are as follows. We demonstrated that a random urine portion cannot be reliably used for uromodulin measurement. According to our findings, an optimal option for uromodulin concentration assessment would be 24-h urine collection as the corresponding ICCs appeared to be fairly high, and in cases when

superior accuracy of the measurement is desired evaluation over several days is warranted. Furthermore, while being largely a methodological study, it nevertheless provides some mechanistic insights. We observed that in the course of the day urinary excretion of uromodulin closely followed diurnal fluctuations in urine volume. There was no "dilution effect" – drop in concentration with increase in volume of urine – that is usually seen for a number of substances excreted with urine. This phenomenon is suggestive of a role of urine volume in uromodulin shedding which is probably driven/facilitated by tubular flow. Our results are thus in agreement with an experimental study showing that urinary excretion of uromodulin followed the increased diuresis and increased in rats with polyuria.[28]

Here we studied healthy individuals; correspondingly, the results might not be generalizable to renal patients as under pathophysiological conditions dysregulation of uromodulin excretion may occur. Therefore, investigation of uromodulin day-to-day and intra-day variability in specifically subjects with compromised kidney function would be of interest. However, a study of patients with nephrolithiasis, which evaluated uromodulin in three 24-h urine collections, found its excretion to be remarkably constant[29], which is in line with our findings in healthy subjects.

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Part 2

Uromodulin response to renoprotective treatment

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Introduction

Uromodulin (or Tamm-Horsfall protein) is the most abundant tubular protein in the urine of healthy subjects. It is expressed in the kidney, primarily in the thick ascending limb of the loop of Henle with negligible expression elsewhere, and released into urine by proteolytic cleavage.

Uromodulin excretion has been previously associated with renal prognosis. It has been shown that elevated urinary uromodulin levels associate with progressive renal function decline and increased risk for development of chronic kidney disease (CKD)[1]. For better interpretation of urinary uromodulin levels it is important to understand its determinants, in both healthy subjects and renal patients.

Association of genetic variation in the *UMOD* gene with blood pressure and fractional excretion of sodium[2], and the peculiar localization of its expression, i.e. in the distal tubule specifically omitting *macula densa*[3,4], are suggestive of potential involvement of uromodulin in renal sodium handling. In healthy subjects sodium intake affects uromodulin excretion, with urinary uromodulin being positively associated with urinary sodium excretion[2,5]. Whether dietary sodium influences uromodulin excretion also in subjects with renal disease is unknown.

Combination of renin-angiotensin-aldosterone system (RAAS) inhibition by either angiotensin converting enzyme inhibition (ACEi) or angiotensin receptor blockade (ARB) (or both, dual RAAS blockade) and low sodium diet constitutes an efficient strategy for renoprotection in patients with proteinuric kidney disease.

We studied uromodulin excretion in renal patients receiving renoprotective treatment. We aimed to evaluate the impact of renoprotective therapy by means of sodium intervention with sodium restricted diet and RAAS inhibition on uromodulin urinary levels in patients with proteinuric kidney disease. To this purpose, we performed a post-hoc analysis of a previously published multicenter randomized double-blind placebo controlled cross-over trial[6]. Our study question was whether uromodulin excretion is affected by intensification of renoprotective treatment in CKD patients.

Methods

Study population and design

The protocol was described in detail elsewhere[6]. Briefly, 52 patients with non-diabetic nephropathy were enrolled and completed the study between April 2006 and October 2009 in three medical centers in the Netherlands. All patients were treated during four 6-week periods, in random order (cross-over design), with ARB at maximal recommended dose (valsartan 320 mg/day) or placebo, each combined with, consecutively, a low

sodium diet (target 50 mmol Na^+/day) and a regular sodium diet (target 200 mmol Na^+/day), on a background of ACE inhibition at a maximal recommended dose (lisinopril 40 mg/day) during the entire study. The drug interventions were double-blind, the dietary interventions were open-label. At the end of each 6-week treatment period patients collected 24-h urine. A flowchart of the study design is presented in **Figure 1**.

Uromodulin measurement

We measured uromodulin concentration in aliquots of the 24-h urine collections available at storage, using a commercially available enzyme-linked immunosorbent assay kit, Tamm-Horsfall Glycoprotein ELISA (MD Bioproducts, St. Paul, MN), according to the manufacturer's instructions.

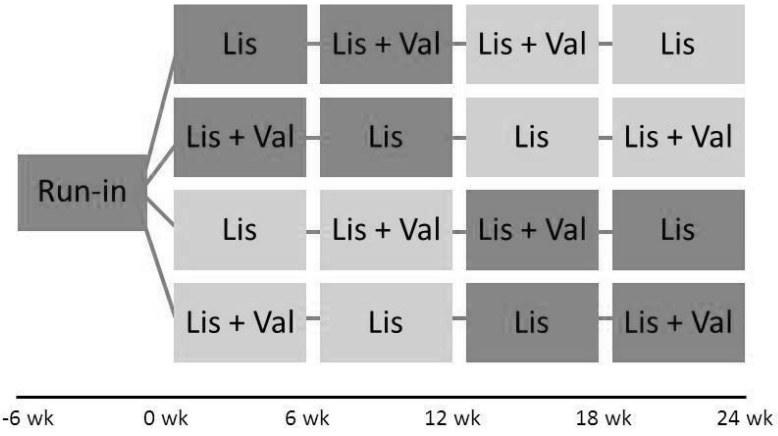

Figure 1. Study design flowchart.

During a 6-week run-in period, patients received ACE inhibition at maximal dose (lisinopril 40 mg/day) and stopped all other renin-angiotensin-aldosterone system blockers. Additional antihypertensive drugs such as β blockers, α blockers, calcium channel blockers, and diuretics were allowed and kept stable during the study; no dietary intervention took place during the run-in period. Consequently, the patients were treated during four 6-week periods, in random order, with angiotensin receptor blockade at maximal recommended dose (valsartan 320 mg/day) or placebo, each combined with, consecutively, a low sodium diet (target 50 mmol Na^+/day, approximately 3 g NaCl/day) and a regular sodium diet (target 200 mmol Na^+/day, 12 g NaCl/day), on a background of ACE inhibition at a maximal recommended dose (lisinopril 40 mg/day) during the entire study. At the end of each 6-week treatment period patients collected 24-h urine.

Lis, lisinopril; Val, valsartan; dark gray background color indicates regular sodium diet, light gray - low sodium diet.

Statistical analysis

Uromodulin 24-h excretion (mg/24h) was calculated as uromodulin concentration × 24-h urine volume. Before statistical testing, uromodulin excretion values were natural log transformed to approximate normality of distribution. We used paired t-test (which account for the same patients providing data for both treatment groups) to determine the effect of treatment regimen. Six comparisons were made:

1) lisinopril *versus* lisinopril plus valsartan,
2) lisinopril *versus* lisinopril plus low sodium diet,
3) lisinopril *versus* lisinopril plus valsartan plus low sodium diet,
4) lisinopril plus valsartan *versus* lisinopril plus low sodium diet,
5) lisinopril plus valsartan *versus* lisinopril plus valsartan plus low sodium diet,
6) lisinopril plus low sodium diet *versus* lisinopril plus valsartan plus low sodium diet.

To adjust for multiple testing, we set the significance threshold to 0.0083 (0.05/6, Bonferroni correction). To verify the absence of carry-over effects, we performed a linear mixed model analysis with uromodulin excretion as a dependent variable, participants as a random factor, treatment, sequence and the interaction term treatment*sequence as fixed factors.

Results and Discussion

Uromodulin excretion was not significantly affected by either mono or dual RAAS blockade or dietary sodium, as there were no statistically significant differences in uromodulin 24-h excretion between the treatment regimens: lisinopril *versus* lisinopril plus valsartan (p=0.822); lisinopril *versus* lisinopril plus low sodium diet (p=0.219); lisinopril *versus* lisinopril plus valsartan plus low sodium diet (p=0.065); lisinopril plus valsartan *versus* lisinopril plus low sodium diet (p=0.265); lisinopril plus valsartan *versus* lisinopril plus valsartan plus low sodium diet (p=0.026); lisinopril plus low sodium diet *versus* lisinopril plus valsartan plus low sodium diet (p=0.189).

However, we observed a trend for decreased uromodulin urinary excretion during low sodium as compared to high sodium diet (**Table 1**, **Figure 2**). Such a direction of the effect is in line with the previous studies. It had been previously shown that sodium intake affects uromodulin excretion in healthy subjects[2,5]. Also in a recent report urinary uromodulin excretion was positively correlated with urinary sodium excretion[7]. Our study thus demonstrated that also in CKD patients urinary uromodulin is influenced by dietary sodium.

Lis 16.0±9.9	Lis + Val 16.3±13.0	Lis + Val 12.0±6.2	Lis 12.8±7.0
Lis + Val 18.5±14.6	Lis 19.0±14.0	Lis 14.9±12.0	Lis + Val 16.3±17.3
Lis 16.3±13.4	Lis + Val 16.8±11.9	Lis + Val 18.6±18.7	Lis 22.6±15.5
Lis + Val 14.3±8.1	Lis 9.9±6.4	Lis 13.7±11.0	Lis + Val 21.7±15.6

0 wk 6 wk 12 wk 18 wk 24 wk

Table 1. Uromodulin 24-h excretion per treatment period.

Data are presented as mean±S.D. of untransformed values

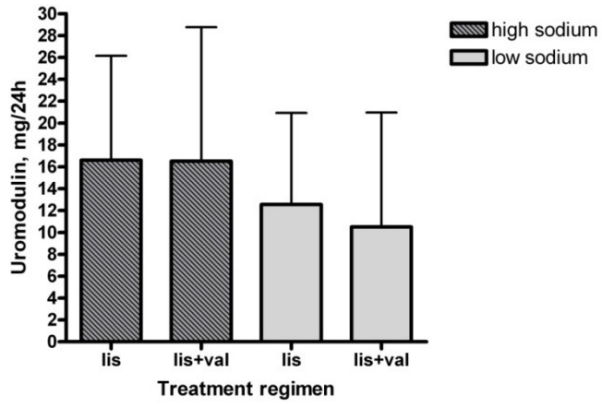

Figure 2. Uromodulin 24-h excretion by treatment regimen.

The interventions are designated as follows: lis, lisinopril; lis+val, dual RAAS blockade with lisinopril and valsartan. The columns represent medians of untransformed values, the error bars – interquartile range.

The fact that uromodulin response to sodium load is preserved in individuals with impaired renal function is of interest and large importance, since it then constitutes a potential target for intervention.

High urinary uromodulin content is assumed to be harmful. First, it is a prognostic factor, as elevated uromodulin levels have been shown to be associated with increased risk for development of CKD[1]. Second, uromodulin is thought to play a direct pathogenetic role in renal disease by promoting inflammation-mediated tubulointerstitial damage[8-10]. Therefore, dietary sodium restriction might represent a plausible intervention to slow down progression of CKD through lowering urinary uromodulin.

Angiotensin converting enzyme inhibition has previously been reported to decrease uromodulin excretion[11]. In our study dual RAAS inhibition with angiotensin receptor blockade on top of ACEi did not affect urinary uromodulin levels. This might be attributed

to the fact that maximal decrease of uromodulin has already been reached by lisinopril, and addition of valsartan did not further reduce excretion of uromodulin. In this respect, it would have been of interest to measure a baseline (without ACEi) uromodulin excretion. However, it is not feasible since the protocols of treatment of patients with proteinuric kidney disease include ACE inhibitors.

A certain limitation of our study that needs to be mentioned is that there were no wash-out periods between the treatments. However, the half-life of the used interventions is rather short (lisinopril 12.6 hours, valsartan 9 hours, low sodium diet <1 week). In addition, the treatments were given in random order. This allowed to design the study without wash-out periods. The linear mixed model analysis confirmed that there was no effect of order of the treatment regimens.

We studied changes of uromodulin content in urine, assuming that urinary uromodulin reflects renal uromodulin content. However, uromodulin urinary excretion might not equal its tissue expression. During high dietary salt intake, elevated excretion of uromodulin could potentially be due to its increased synthesis in the kidney, increased shedding into urine, or both. Our study design did not allow to discriminate between these mechanisms. However, an experimental study performed in rats showed increased expression of uromodulin in the kidney in response to increased dietary sodium intake[12]. Studies involving simultaneous assessment of kidney and urinary uromodulin are warranted to investigate the mechanisms by which sodium intake affects uromodulin excretion.

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Chapter 4

UMOD as a susceptibility gene for end-stage renal disease

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Abstract

In recent genetic association studies, common variants including rs12917707 in the *UMOD* locus have shown strong evidence of association with eGFR, prevalent and incident chronic kidney disease and uromodulin urinary concentration in general population cohorts. The association of rs12917707 with end-stage renal disease (ESRD) in a recent case-control study was only nominally significant.

To investigate whether rs12917707 associates with ESRD, graft failure (GF) and urinary uromodulin levels in an independent cohort, we genotyped 1142 ESRD patients receiving a renal transplantation and 1184 kidney donors as controls. After transplantation, 1066 renal transplant recipients were followed up for GF. Urinary uromodulin concentration was measured at median [IQR] 4.2 [2.2-6.1] yrs after kidney transplantation.

The rs12917707 minor allele showed association with lower risk of ESRD (OR 0.89 [0.76-1.03], p=0.04) consistent in effect size and direction with the previous report (Böger *et al*, PLoS Genet 2011). Meta-analysis of these findings showed significant association of rs12917707 with ESRD (OR 0.91 [0.85-0.98], p=0.008). In contrast, rs12917707 was not associated with incidence of GF. Urinary uromodulin concentration was lower in recipients-carriers of the donor rs12917707 minor allele as compared to non-carriers, again consistent with previous observations in general population cohorts.

Our study thus corroborates earlier evidence and independently confirms the association between *UMOD* and ESRD.

Introduction

Chronic kidney disease (CKD) is a significant and increasing global challenge for public health. It affects ≈10% of the general population in industrialized nations, incurring high morbidity and mortality, and posing a substantial financial burden to the health care systems [1-3]. It is a complex, multifactorial disorder with an important genetic component. Identification of the genetic variants involved in its susceptibility and progression to end-stage renal disease (ESRD) will improve our understanding of biological mechanisms underlying renal function and will ultimately lead to development of novel tools for diagnosis, prevention, prediction and treatment [4-6].

Recent advances in genome-wide association (GWA) studies of kidney disease resulted in discovery of several genes. Among them a prominent place is taken by *UMOD* which has been reproducibly identified in multiple cohorts as one of the top loci associated with renal function parameters [7-10]. Several GWA studies highlighted a region upstream from the *UMOD* gene containing rs12917707 and several other SNPs in high linkage disequilibrium (LD). The mentioned LD block was repeatedly shown to be associated with prevalent and incident CKD, and also uromodulin urinary concentration. All the studies showed a consistent trend of association of the rs12917707 minor allele with lower risk of CKD [7, 11-15], and the minor alleles of SNPs in perfect LD with rs12917707, rs4293393 and rs13333226, were associated with lower urinary uromodulin levels [11, 15].

A recent study examined the role of rs12917707 genotype in risk for a more severe renal phenotype, ESRD, with the minor allele again showing a protective effect: OR [95% CI] 0.92 [0.86-1.0] [14]. However, the level of statistical significance was only nominal (p=0.04), warranting further investigation to confirm the association of the UMOD variants with kidney damage phenotypes.

We thus analyzed the association of rs12917707 with ESRD and with graft failure (GF) after kidney transplantation, and investigated the effect of rs12917707 genotype on urinary uromodulin levels. First, we performed a case-control study where cases were 1142 ESRD patients receiving transplantation and controls were 1184 kidney donors (a flowchart of the participants selection is shown in **Figure 1**). Second, to analyze whether *UMOD* affects long-term kidney transplant function, we performed a survival association analysis of donor rs12917707 genotype impact on incidence of GF in 1066 renal transplant recipients.

The *UMOD* gene expression product is uromodulin, also known as Tamm-Horsfall protein, which is excreted with urine, easily measurable [16-18] and thus presents a perfect intermediate phenotype for genetic association research. As the *UMOD* gene is expressed exclusively in the kidney, it was assumed that it was kidney genotype that was associated with urinary uromodulin in the previous reports [11-13, 15]. To prove it, we aimed to investigate whether this association holds after the kidney is transplanted.

Methods & Materials

Study population

This study was conducted in the REGaTTA cohort [19, 20]. Briefly, from all renal transplantations carried out in our center between 1993 and 2008 we included 1142 first graft recipients and 1186 donors (1066 matched donor-recipient pairs) for the present genetic study. The exclusion criteria were: re-transplantation, combined kidney/pancreas or kidney/liver transplantation, technical problems, absence of DNA and loss of follow-up. A flowchart of the study participants selection is shown in **Figure 1**. After transplantation the recipients were followed up for median [IQR] 5.5 [2.9-8.8] years and immunosuppression regimen, clinical and laboratory parameters, and time to GF were documented. GF was defined as return to dialysis or re-transplantation and was censored for death with a functioning graft. Patients characteristics, transplantation-related parameters, clinical and laboratory data were retrieved from medical records. The Institutional Review Board of the University Medical Center Groningen approved the study protocol. Informed consent was given by all transplant recipients and living donors. For deceased donors, with research carried out after the organ removal and implantation, no consent was required. According to Dutch law general consent for organ donation and transplantation includes consent for research projects. The study was conducted according to the principles of the Declaration of Helsinki. All the genetic and clinical data were anonymized prior to analyses.

DNA isolation and genotyping

DNA was extracted from peripheral whole blood (in recipients and living donors) or lymph nodes/spleen lymphocytes (in deceased donors) using a commercial kit following the manufacturer's instructions, transferred into 2 ml Eppendorf tubes and stored at -20°C. Absorbance at 260nm was measured with NanoDrop spectrophotometer (ND-1000, NanoDrop Technologies) and DNA concentration was calculated by the NanoDrop nucleic acid application module. As a measure of DNA purity 260/280 and 260/230 absorbance ratios were assessed. Where samples failed to meet the minimum DNA concentration and purity recommended for Illumina genotyping, repeated isolation attempts were made. Genotyping of the rs12917707 SNP in the *UMOD* locus was performed using the Illumina VeraCode GoldenGate assay kit (Illumina, San Diego, CA, USA), according to the manufacturer's instructions. Genotype clustering and calling were performed using BeadStudio Software (Illumina). Genotyping was unsuccessful in two individuals.

Uromodulin measurement

For 282 outpatient renal transplant recipients at median [IQR] 4.2 [2.2-6.1] years post-transplant, 24 h urine samples were available at storage. Uromodulin concentration was measured by a commercially available enzyme-linked immunosorbent assay kit (MD Bioproducts, St. Paul, MN, Tamm-Horsfall Glycoprotein ELISA, catalog number M036020) according to the manufacturer's instructions. For this assay, the interassay coefficient of variation is 10.5% at a mean concentration of 21.8 ng/mL and 12.2% at 95.1 ng/mL; the sensitivity is less than 0.75 ng/mL. The principle of the assay is based on a colorimetric sandwich immunoassay utilizing a polyclonal antibody against human uromodulin as the capture antibody and a biotinylated polyclonal antibody against human uromodulin as the detection antibody. Immediately after the ELISA procedure absorbance reading was performed at 450 nm. A standard curve was generated with each set of samples by reducing the data using 4-parameter logistic curve fit. Uromodulin concentration was calculated from the standard curve.

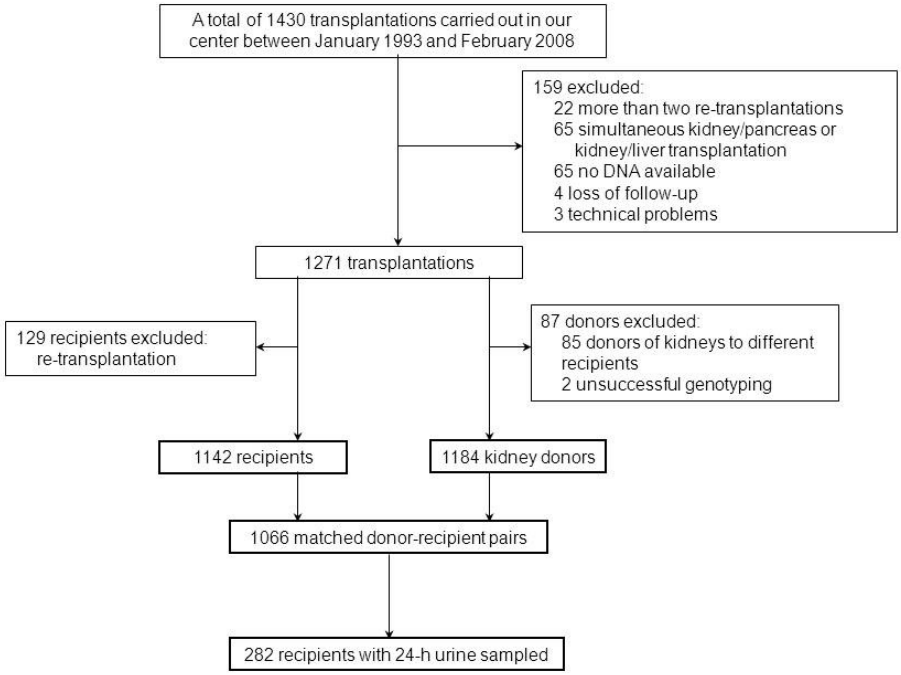

Figure 1. A flowchart of the study participants selection

Statistical analysis

Analyses were performed with PASW Statistics 18.0 (SPSS Inc., Chicago, IL) and PLINK v1.07 (S. Purcell, http://pngu.mgh.harvard.edu/purcell/plink/ [21]). QUANTO v1.2.4 (http://hydra.usc.edu/gxe/) and PASS v11 were used for power estimation.

Deviation from Hardy-Weinberg equilibrium was tested in donors. Two individuals (donors), in which genotyping of rs12917707 was unsuccessful, were excluded and subsequent statistical analyses were performed on a final sample of 2326 subjects in a case-control design (1142 recipients vs 1184 donors) and 1066 matched donor-recipient pairs in a longitudinal design. Genotype-phenotype associations were tested under an additive genetic model and results (regression coefficients and p-values) are reported per copy of the minor allele.

Since we tested the hypothesis that the minor rs12917707 allele is associated with reduced risk, such a priori directional prediction allowed us to assume statistical significance at a one-sided p=0.05. Also in the reference paper [14] one-sided p values were presented. With one-sided p=0.05, assuming an additive genetic model and MAF of 20%, we had approximately 90% and 40% power to detect an OR of 0.8 and 0.9, respectively, in the ESRD case-control analysis, and 47% and 18% power to detect a HR of 0.8 and 0.9, respectively, in the longitudinal analysis of graft survival.

As in 164 (14%) cases transplantation was performed from living blood-related donors, the PLINK DFAM algorithm was used to account for relatedness in the case-control analysis.

We performed a fixed effects inverse variance meta-analysis to combine the results of our case-control study and the previously published one [14].

The effect of genotype on graft survival was investigated with Cox regression analysis including as covariates known predictors of GF (donor and recipient age and sex, donor type, ischemia times, delayed graft function and acute rejection episodes history, immunosuppression regimen).

To study association between genotype and uromodulin urinary levels after transplantation, due to small number of individuals homozygote for the minor allele, genotypes were combined into two groups: minor allele carriers (heterozygotes and homozygotes for the minor allele) and non-carriers (homozygotes for the ancestral allele). Statistical significance of differences between the groups was tested with a Mann-Whitney U test.

Results

Patient characteristics are presented in **Table 1**.

There was no deviation from Hardy-Weinberg equilibrium in controls (p=0.49). The rs12917707 minor allele frequency (MAF) in the overall sample was 17.3%, comparable to HapMap data and previous publications [7, 8, 14]. The MAF was 18.2% and 16.5% in kidney donors and ESRD patients, respectively. In the additive genetic model, adjusted for age, sex and case-control relatedness, OR [95% CI] for ESRD was 0.89 [0.76-1.03] per copy of the minor allele, one-sided p=0.04, which is direction-consistent with the previously published association results [14]. A meta-analysis of our results and those of the abovementioned study [14] showed a significant association of rs12917707 with ESRD: OR [95% CI] 0.91 [0.85-0.98], p=0.008 (**Figure 2**).

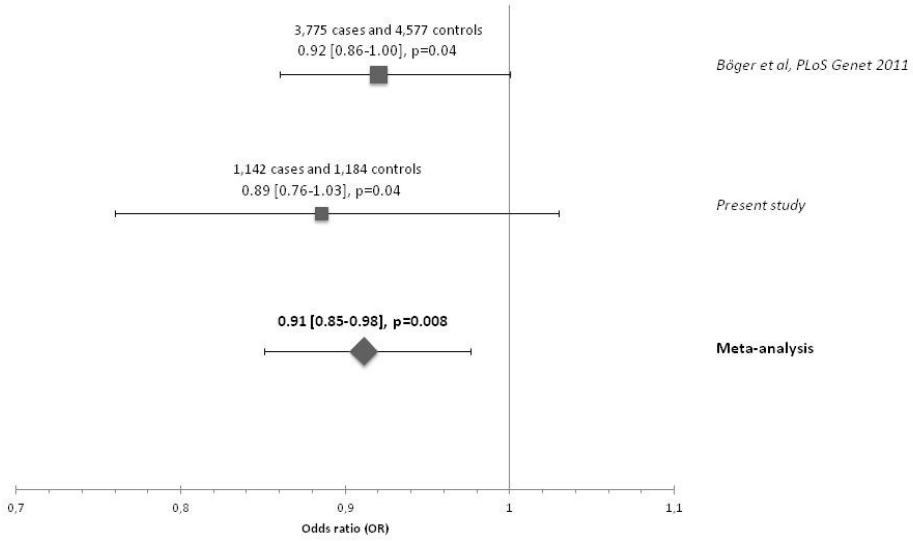

Figure 2. Meta-analysis forest plot.

There was no interaction between rs12917707 and age or sex (p=0.92 and p=0.97, respectively). We did not observe association between rs12917707 genotype and any of the underlying etiology of ESRD (**Table S1**).

Table 1. Patient characteristics.

ESRD patients, n=1142	
Age, years	47.7±13.5
Sex: male, n (%)	662 (58.0)
Primary disease:	
- glomerulonephritis, n (%)	242 (21.2)
- autosomal dominant polycystic kidney disease, n (%)	155 (13.6)
- pyelonephritis, n (%)	128 (11.2)
- renal vascular disease, n (%)	110 (9.6)
- IgA nephropathy, n (%)	89 (7.8)
- diabetes types I and II, n (%)	47 (4.1)
- other/uncertain etiology, n (%)	371 (32.5)
Kidney donors, n=1184	
Age, years	44.5±14.3
Sex: male, n (%)	602 (50.8)
Living donors, n (%)	282 (23.8)
- from which related donors, n (%)	164 (58.2)
Matched donor-recipient pairs, n=1066	
Recipient age, years	48.1±13.5
Recipient sex: male, n (%)	620 (58.2)
Donor age, years	44.6±14.3
Donor sex: male, n (%)	540 (50.7)
Living donors, n (%)	261 (24.5)
Cold ischemia time, hours	17.4 [9.0-23.0]
Total warm ischemia time, minutes	40.0 [34.0-51.0]
Delayed graft function, n (%)	332 (31.1)
Post-transplant follow-up duration, years	5.5 [3.0-8.7]
Acute organ rejection episodes history, n (%)	368 (34.5)
Death-censored graft failure, n (%)	172 (16.1)
Death with a functioning graft, n (%)	182 (17.1)
Renal transplant recipients with urine available, n=282	
Age, years	52.1±12.3
Sex: male, n (%)	152 (53.9)
Urine collection time point, years after transplantation	4.2 [2.2-6.1]

Continuous normally distributed variables are presented as means±SD, non-normally distributed – as medians [IQR].

During a median [IQR] 5.5 [2.9-8.8] years of follow-up, 172 (16.1%) cases of death-censored GF occurred and 183 (17.1%) patients died with a functioning graft. Donor kidney rs12917707 MAF was higher in subjects with a functioning graft as compared to cases that suffered death-censored GF (18.7% vs 17.2%, respectively). There was no significant association between donor rs12917707 and GF as in a univariate Cox regression analysis hazard ratio (HR) [95% CI] for GF was 0.92 [0.69-1.23], p=0.57.

A multivariate Cox regression model adjusted for known predictors of graft survival (**Table S2**) showed a HR [95% CI] 0.96 [0.72-1.28], p=0.76. Exclusion of cases with ≤1 year graft survival use did not change the results. Recipient rs12917707 also was not associated with GF (HR [95% CI] 1.04 [0.78-1.38], p=0.788).

In a subset of renal transplant recipients (n=282), in which 24-h urine collected at median [IQR] 4.2 [2.2-6.1] years post-transplant was available for uromodulin measurements, donor rs12917707 genotypes distribution was 4 / 94 / 184, correspondingly, MAF was 18.0%. Uromodulin concentrations ranged from 0.3 to 42.6 µg/ml, median [IQR] 8.6 [5.8-13.3] µg/ml, and were significantly lower in carriers of the rs12917707 minor allele in the donor genotype as compared to non-carriers: 7.3 [5.4-10.8] and 9.4 [6.4-15-6] µg/ml, respectively (p=0.007) [**Figure S1**]. Recipient rs12917707 was not associated with uromodulin urinary levels (p=0.43).

Discussion

Thus, we have independently confirmed the association between *UMOD* rs12917707 and ESRD in a large case-control study. It was of similar direction and magnitude as previously reported [14], and in a meta-analysis of our results and the cited data convincing statistical significance was reached.

We did not find an association between rs12917707 genotype and etiology of ESRD. This suggests a universal, non-specific effect of the SNP on renal function decline irrespectively of underlying primary disease. A previous study described interaction between *UMOD* variant rs4293393 (in perfect LD with rs12917707) and age [12]. We, however, did not observe interaction with age or sex in our population.

Importantly, the observed relationship of the *UMOD* variant with ESRD in native kidneys did not translate into association with renal function loss in the transplanted kidney as we did not find an association between the donor rs12917707 and GF. Although the SNP effect on GF was direction-consistent with the case-control analysis and suggestive of a protective trend, the results were not statistically significant. It might indicate true absence of an association and differential involvement of *UMOD* in the pathophysiology of native and transplanted kidneys, or point to the fact that our longitudinal study was underpowered to detect the genetic effect due to the moderate sample size.

Remarkably, uromodulin urinary levels in patients after renal transplantation were associated with donor *UMOD* rs12917707 genotype. The subset of transplant recipients, in which urinary uromodulin was studied, was representative of the whole sample in terms of MAF and genotypes distribution. Uromodulin concentrations were significantly lower in recipients-carriers of the donor rs12917707 minor allele as compared to non-carriers. Thus, the genetic effect on uromodulin urinary level that was previously found in the native kidneys was reproduced in the transplanted kidneys, with similar direction of effect. This implies that it is indeed the *UMOD* genotype of the kidney that associates with uromodulin production.

Several arguments support the genetic analysis of a kidney transplant cohort. First, a case-control study with kidney donors, instead of the general population, as controls may have augmented statistical power to reveal the subtle genetic effects expected from common variants. Second, a transplant population provides the opportunity to study renal function loss in both native and transplanted kidneys through investigation of ESRD before and GF after transplantation. Finally, uniqueness and elegance of a transplantation setting is that it enables to test effects of both recipient and donor genotype on phenotype and thus discriminate between local (intra-renal) and systemic (extra-renal) processes.

The strengths of our study include the cohort's size, its wide spectrum of underlying primary kidney disease and the specific design. However, some limitations deserve to be mentioned. Our longitudinal study may have been underpowered to detect significant SNP effect on GF. Power limitations also exist for the analysis of specific ESRD etiologies. Further, the analysis of the risk for ESRD is cross-sectional and needs to be confirmed by longitudinal studies studying incident ESRD. Unfortunately, the design and performance of such studies in CKD patients is challenging, and thus these are only emerging [22-24]. We did not have information on patients ethnicity, however, a reliable estimate for an average patient population in our region is that over 90% of the individuals are of European ancestry. Subsequently, our results are not generalizable to other ethnicities. Urinary uromodulin in renal transplant recipients was measured at different time points ranging from 1 to 9 years after transplantation. For those patients with presence of residual native kidney function, we cannot exclude a possible confounding effect of recipient rs12917707 genotype on urinary uromodulin concentration. However, this would have biased results to a null effect, while we have detected a significant association.

In summary, we have independently confirmed the association between genetic variation at the *UMOD* locus and ESRD. Also, donor kidney genotype was significantly associated with urinary uromodulin concentration in renal transplant recipients providing evidence that genetic make-up of the kidney determines this intermediate phenotype. Further research, including targeted sequencing of the region, bioinformatic analyses and functional experiments, is required to unravel the mechanisms by which common genetic variation at *UMOD* cause kidney disease.

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Supplementary Material

Table S1. Distribution of primary diseases in relation to UMOD rs12917707 genotype in ESRD patients.

	***UMOD* rs12917707**			*P* value
	0	**1**	**2**	
N	799	310	33	
Glomerulonephritis, n (%)	168 (21.0)	69 (22.3)	5 (15.2)	0.623
Polycystic kidney disease, autosomal dominant (adult) form, n (%)	111 (13.9)	37 (11.9)	7 (21.2)	0.298
Pyelonephritis, n (%)	91 (11.4)	35 (11.3)	4 (12.1)	0.990
Renal vascular disease, n (%)	81 (10.1)	26 (8.4)	3 (9.1)	0.671
IgA nephropathy, n (%)	61 (7.6)	26 (8.4)	2 (6.1)	0.853
Diabetes type I and II, n (%)	33 (4.1)	14 (4.5)	0 (0.0)	0.462

Digits 0-2 designate number of the rs12917707 minor allele copies per genotype

Table S2. Predictors of GF (univariate Cox regression analysis) in 1066 renal transplant recipients followed up for a median [IQR] 5.5 [2.9-8.8] years after transplantation

	HR	95% CI	*P* value
Recipient age at transplantation	0.99	0.98-1.00	0.037
Recipient sex: male	1.11	0.82-1.51	0.495
Donor age	1.02	1.01-1.03	0.002
Donor sex: male	0.98	0.73-1.33	0.907
Donor type: living vs deceased	0.54	0.35-0.84	0.006
Cold ischemia time	1.03	1.01-1.04	0.001
Total warm ischemia time	1.02	1.01-1.03	0.001
Delayed graft function	3.83	2.83-5.19	<0.001
Acute rejection episodes history	1.87	1.38-2.52	<0.001

HR, hazard ratio; CI, confidence intervals.

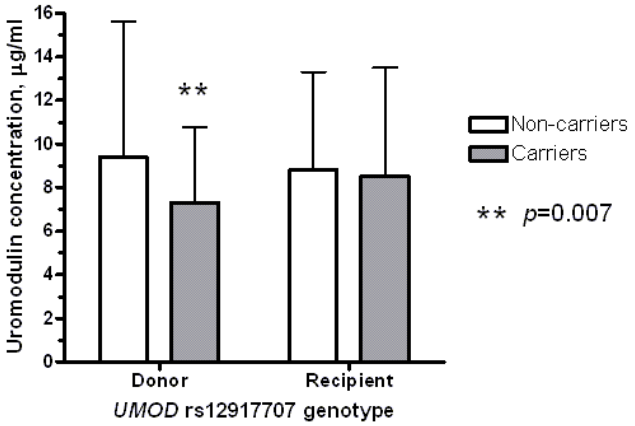

Figure S1. Uromodulin urinary concentration in 282 renal transplant recipients at 4.2 [2.2-6.1] yrs post-transplant by donor and recipient genotype stratified by presence (carriers) or absence (non-carriers) of UMOD rs12917707 minor allele.

Columns represent respective medians of values, error bars – interquartile range. P=0.007, Mann-Whitney U-test.

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Chapter 5

CUBN as a novel locus for end-stage renal disease: insights from renal transplantation

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Abstract

Chronic kidney disease (CKD) is a complex disorder. As genome-wide association studies identified cubilin gene *CUBN* as a locus for albuminuria, and urinary protein loss is a risk factor for progressive CKD, we tested the hypothesis that common genetic variants in *CUBN* are associated with end-stage renal disease (ESRD) and proteinuria.

First, a total of 1142 patients with ESRD, admitted for renal transplantation, and 1186 donors were genotyped for SNPs rs7918972 and rs1801239 (case-control study). The rs7918972 minor allele frequency (MAF) was higher in ESRD patients comparing to kidney donors, implicating an increased risk for ESRD (OR 1.39, p=0.0004) in native kidneys.

Second, after transplantation recipients were followed for 5.8 [3.8-9.2] years (longitudinal study) documenting ESRD in transplanted kidneys – graft failure (GF). During post-transplant follow-up 92 (9.6%) cases of death-censored GF occurred. Donor rs7918972 MAF, representing genotype of the transplanted kidney, was 16.3% in GF vs 10.7% in cases with functioning graft. Consistently, a multivariate Cox regression analysis showed that donor rs7918972 is a predictor of GF, although statistical significance was not reached (HR 1.53, p=0.055). There was no association of recipient rs7918972 with GF. Rs1801239 was not associated with ESRD or GF.

In line with an association with the outcome, donor rs7918972 was associated with elevated proteinuria levels cross-sectionally at 1 year after transplantation.

Thus, we identified *CUBN* rs7918972 as a novel risk variant for renal function loss in two independent settings: ESRD in native kidneys and GF in transplanted kidneys.

Introduction

Chronic kidney disease (CKD) is a complex multifactorial disorder with an important genetic component [1-3]. A recent genome-wide association study (GWAS) identified the cubilin gene *CUBN* as a locus for albuminuria: a missense single-nucleotide polymorphism (SNP) rs1801239 (Ile2984Val) in this gene was associated with elevated urinary albumine-to-creatinine ratio and microalbuminuria in both the general population and in diabetic patients [4].

As albuminuria is a risk factor for progression of CKD up to end stage renal disease (ESRD) [5], we hypothesized that genetic variation in *CUBN* is associated with development of ESRD. To test this hypothesis we genotyped patients with ESRD, admitted for renal transplantation, with their donors as a control population, for SNPs in the *CUBN* locus and followed the recipients after transplantation documenting clinical parameters and occurrence of graft failure (GF).

Two *CUBN* SNPs were genotyped in our study: the previously published rs1801239 and a tagSNP rs7918972. The latter was selected based on its linkage disequilibrium with 9 other SNPs thus covering more variability in the locus and taking into account that one of the linked polymorphisms is a coding missense variant which might potentially be functional. Another selection criterion was the minor allele frequency (MAF); we targeted a lower part of the common variability range, with MAFs between 10 and 15%.

Within this cohort we performed essentially two independent analyses: 1) ESRD patients admitted for renal transplantation versus kidney donors (extreme case-control study) – to test for association with ESRD in native kidneys; and 2) long-term post-transplant follow-up for GF in the recipients (longitudinal study) – for association with ESRD in the transplanted kidney.

We also tested association of the *CUBN* SNPs with 24-h total urinary protein excretion as an intermediate phenotype.

Methods & Materials

Study population

From all renal transplantations carried out in our center between 1993 and 2008 we retrospectively selected 1142 first graft recipients and 1186 donors for the present genetic study. The exclusion criteria were: cases of re-transplantation, combined kidney/pancreas or kidney/liver transplantation, technical problems, absence of DNA and loss of follow-up. A flowchart of the study participants selection is shown in the **Figure 1**. After transplantation the recipients were followed up and immunosuppression regimen, clinical and laboratory parameters, and time to GF were documented. GF was defined

as return to dialysis or re-transplantation and was censored for death with a functioning graft. Cases with post-transplant graft survival <1 year were excluded from the analyses, to decrease heterogeneity in the sample, as graft loss < 1 year is to an important extent due to acute complications, such as technical surgical problems, delayed graft function and/or acute rejection episodes, whereas we wanted to focus on the process of chronic transplant dysfunction. Donor and recipient characteristics, transplantation-related parameters and clinical data (24h urinary protein excretion, blood pressure, renal function) were retrieved from medical records. The Institutional Review Board of the University Medical Center Groningen approved the study protocol. Written informed consent was given by all recipients and living donors. For deceased donors, with research carried out after the organ removal and implantation, no consent was required. According to Dutch law general consent for organ donation and transplantation includes consent for research projects. The study was conducted according to the principles of the Declaration of Helsinki. All the genetic and clinical data were anonymized prior to analyses.

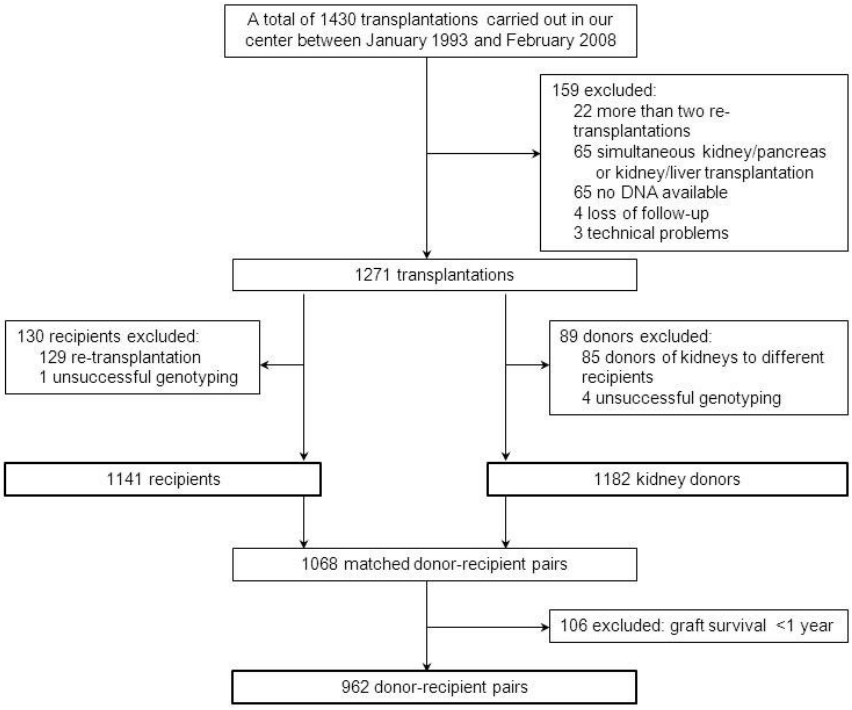

Figure 1. A flowchart of the study participants selection

DNA isolation, tagSNP selection and genotyping

DNA was extracted from peripheral whole blood (in recipients and living donors) or lymph nodes/spleen lymphocytes (in deceased donors) using a commercial kit following the manufacturer's instructions, transferred into 2 ml Eppendorf tubes and stored at -20°C. Absorbance at 260nm was measured with NanoDrop spectrophotometer (ND-1000, NanoDrop Technologies) and DNA concentration was calculated by the NanoDrop nucleic acid application module. As a measure of DNA purity 260/280 and 260/230 absorbance ratios were assessed. Where samples failed to meet the minimum DNA concentration and purity recommended for Illumina genotyping, repeated isolation attempts were made.

Two SNPs in the *CUBN* locus were genotyped: missense (Ile2984Val) rs1801239 and rs7918972. The latter is a tagSNP in the *CUBN* intron, which was selected using Genome Variation Server v5.11 (Seattle SNPs Program for Genomic Applications). This program utilizes the LDSelect algorithm [6]. All the SNPs within the *CUBN* gene including 500 bases at the gene flanking regions were submitted to the selection procedure. The following parameter settings were used: HapMap-CEU population (unrelated only, no HapMap 3), monomorphic sites excluded, r^2 threshold 0.8, minimal genotype coverage for tagSNPs 85%. Further, for our study we considered SNPs with MAFs 10-15%, tagging as many other variants as possible including the missense ones. Rationale for the arbitrary MAF cut-off was based on the general expectation that rarer variants have a slightly higher likelihood to be causal and may confer stronger effects. At the same time, as power to detect such effects depends on sample size, we were constrained by the moderate sample size of our study. That is why we set the cut-off in the range of 10-15%. Using these settings, the SNP rs7918972 was the best tagSNP meeting all our criteria (minimal MAF – 10%, maximal number of the tagged SNPs – 9, tagging a missense variant) and therefore was ultimately chosen for this study. This SNP is in strong linkage disequilibrium with intronic SNPs rs4088454, rs7897625, rs7897716, rs7898076, rs11254232, rs11254238, rs7897442, rs7897705 and missense (Asn3552Lys) rs1801232, all of which map to the *CUBN* locus. The LD structure of the studied *CUBN* SNPs is shown in the **Supplemental Figure S1**.

Genotyping of the selected SNPs was performed using the Illumina VeraCode GoldenGate assay kit (Illumina, San Diego, CA, USA), according to the manufacturer's instructions. Genotype clustering and calling were performed using BeadStudio Software (Illumina). In five individuals genotyping was unsuccessful.

Statistical analysis

Analyses were performed with PASW Statistics 18.0 (SPSS Inc., Chicago, IL) and PLINK v1.07 (S. Purcell, http://pngu.mgh.harvard.edu/purcell/plink/) [7]. QUANTO v1.2.4 (http://hydra.usc.edu/gxe/) and PASS v11 were used for power estimation. PolyPhen2 [8] was used to predict functional consequences of the missense SNP. The studied *CUBN* SNPs LD structure plot was generated with SNAP v2.2 [9].

As a routine data quality control, alleles frequencies, Hardy-Weinberg equilibrium and case/control differential missingness were tested for. Subsequent statistical analyses were performed on a final sample of 2323 subjects in a case-control design (1141 recipients vs 1182 donors) and 962 renal transplant recipients in a longitudinal design. With two-sided p=0.05, assuming an additive genetic model and MAF of 10-15%, we had 57% and 99% power to detect an OR of 1.2 and 1.4, respectively, in the ESRD case-control analysis, and 43% and 87% power to detect a HR of 1.5 and 2.0, respectively, in the Cox regression analysis of graft survival.

Genotype-phenotype associations were tested under an additive genetic model and results (regression coefficients and p-values) are reported per copy of the minor allele.

In the case-control analysis, the PLINK DFAM algorithm was used to account for donor-recipient relatedness within living-donor transplantation cases. Interaction between the SNPs was tested with the PLINK *--epistasis* function which includes the interaction term and the marginal effects of the SNPs into the interaction model. Subsequently, stratified logistic regression analyses were performed for each of the three groups of minor allele carriers of both SNPs using the group of non-carriers as the reference.

For the longitudinal study we included cases with post-transplant graft survival ≥1 year. The effect of SNPs on graft survival was investigated with Kaplan-Meier and Cox regression analyses including known predictors of GF (donor and recipient age and sex, donor type, cold and warm ischemia times, immunosuppressive therapy).

Association between genotypes and 24h urinary protein excretion was studied cross-sectionally at 1 year after transplantation assuming stable graft function at this time-point. As proteinuria was considered a left-censored phenotype with 0 values in 24.4% of patients (due to the diagnostic assay detection limit and rounding of routinely reported values), it was analyzed with Tobit regression [10, 11], both univariately and including relevant covariates (age, sex, systolic and diastolic blood pressure).

Results

Main patients characteristics are presented in **Table 1**.

The overall minor allele frequency was 13.1% for rs7918972 and 12.5% for rs1801239. There was no deviation from Hardy-Weinberg equilibrium in controls (p=0.2908 for rs7918972; p=0.4126 for rs1801239). The missing genotypic data fraction was not different between cases and controls (p=1.000 and p=0.625 for rs7918972 and rs1801239, respectively). There was no linkage disequilibrium between rs7918972 and rs1801239 (r^2=0.002, D'=0.059). The missense rs1801232 (Asn3552Lys), tagged by rs7918972, was predicted to be benign by PolyPhen2: score 0.011; sensitivity 0.96; specificity 0.72.

Table 1. Patient characteristics.

ESRD patients, n=1141	
Age, years	48.2±13.5
Sex: male, n (%)	662 (58.0)
Primary disease:	
- glomerulopathies, n (%)	292 (25.6)
- kidney cysts, n (%)	188 (16.5)
- tubulo-interstitial lesions, n (%)	135 (11.8)
- diabetes types I and II, n (%)	47 (4.1)
- renal hypoplasia, n (%)	23 (2.0)
- drug-induced nephritis, n (%)	15 (1.3)
- other/uncertain etiology, n (%)	488 (43)
Kidney donors, n=1182	
Age, years	44.5±14.3
Sex: male, n (%)	603 (51.0)
Living donors, n (%)	282 (23.9)
- from which related donors, n (%)	164 (58.2)
Transplantation, n=962 renal transplant recipients	
Cold ischemia time, minutes	1140 [869-1428]
Total warm ischemia time, minutes	40 [34-50]
Follow-up duration, years	5.8 [3.8-9.5]
Measured GFR at 1 year post-transplant, ml/min	54.8±19.2
Total proteinuria at 1 year post-transplant, g/24h	0.20 [0.05-0.40]
Acute rejection episodes history, n (%)	324 (33.7)
Graft failure, n (%)	92 (9.6)
Death with a functioning graft, n (%)	152 (15.8)

Continuous normally distributed variables are presented as means±SD, non-normally distributed – as medians [IQR].

Case-control study: ESRD patients vs kidney donors

The minor allele frequency (MAF) for rs7918972 was significantly higher in ESRD patients as compared to kidney donors, implicating an increased risk of ESRD: OR [95% CI] 1.39 [1.16-1.65], *p*=0.0004, in an additive model adjusted for age, sex and case-control relatedness (**Table 2**); additional adjustment for diabetes status did not change the results. There was no association between rs7918972 genotype and any of the primary diseases (etiology of ESRD). The MAF for rs7918972 was not different between living and deceased donors and in the latter it was not significantly associated with the cause of death (mortality due to cerebro- or cardiovascular accident vs other reasons). Genotype of rs1801239 was not associated with case/control status or any of the other traits studied.

The effects of the two SNPs were not independent as a case-control test for epistasis revealed an interaction between them (p=5×10-10). A finer analysis showed that the rs7918972 minor allele requires a copy of the rs1801239 minor allele to express its risk phenotype (OR 3.15 [2.21-4.48], p=1.8×10-10), whereas the minor allele of rs1801239 displays protective effect in the absence of rs7918972 minor allele (OR 0.65 [0.52-0.81], p=1.7×10-4) [**Table 3**].

Longitudinal study: post-transplant follow-up

A total of 92 (9.6%) cases of death-censored GF occurred and 151 (15.8%) patients died with a functioning graft during a median [IQR] of 5.8 [3.8-9.5] years of follow-up.

Donor MAF, representing genotype of the transplanted kidney, was higher in subjects that suffered death-censored GF as compared to cases with a functioning graft (16.3% vs 10.7%, respectively). Kaplan-Meier survival analysis revealed worse graft survival (*p*=0.067) for the carriers of the minor allele (**Figure 2**). Consistently, a multivariate Cox regression analysis showed that donor kidney rs7918972 is a predictor of GF yielding a HR of 1.53 [0.99-2.37], *p*=0.055, per copy of the minor allele, in a model adjusted for donor and recipient age and sex, donor type (living vs deceased), ischemia times, immunosuppressive drug use and acute rejection episodes (**Table 4**). In contrast, recipient rs7918972 was not associated with development of GF (HR 1.00, *p*=0.992). Neither donor nor recipient rs1801239 was significantly associated with GF. There was no statistically significant interaction between the two SNPs in the longitudinal analysis of GF. Neither donor nor recipient genotypes were significantly associated with cardiovascular or all-cause mortality during post-transplant follow-up.

Table 2. *CUBN* SNPs in the case-control study of ESRD patients versus kidney donors.

CUBN SNPs		**ESRD patients**, n=1141	**Kidney donors**, n=1182	OR [95% CI] per copy of the minor allele [a]	*p* value[a]
rs7918972	Genotypes, count	21 / 301 / 819	12 / 246 / 924		
	MAF, %	**15.0%**	**11.4%**	**1.39 [1.16-1.65]**	**0.0004**
rs1801239	Genotypes, count	8 / 276 / 857	14 / 266 / 902		
	MAF, %	12.8%	12.4%	1.04 [0.87-1.24]	0.6686

OR, odds ratio; CI, confidence interval. [a]Logistic regression model adjusted for age and sex, with adjustment for case-control relatedness (DFAM algorithm)

Table 3. Interaction between the SNPs in the *CUBN* locus in the case-control study of ESRD patients versus kidney donors.

CUBN SNPs	**rs7918972**		
rs1801239	N of the minor allele copies	0	1 or 2
	0	Reference OR 1.00 *n=1352*	OR 0.93 [0.75-1.15] *p*=0.484 n=407
	1 or 2	OR 0.65 [0.52-0.81] $p=1.7\times10^{-4}$ n=391	OR 3.15 [2.21-4.48] $p=1.8\times10^{-10}$ n=173

Logistic regression model adjusted for age and sex. Odds ratios (OR) [95% confidence intervals] for risk of ESRD, p-values and patients number (n) are presented in relation to simultaneous presence of both minor alleles in genotype.

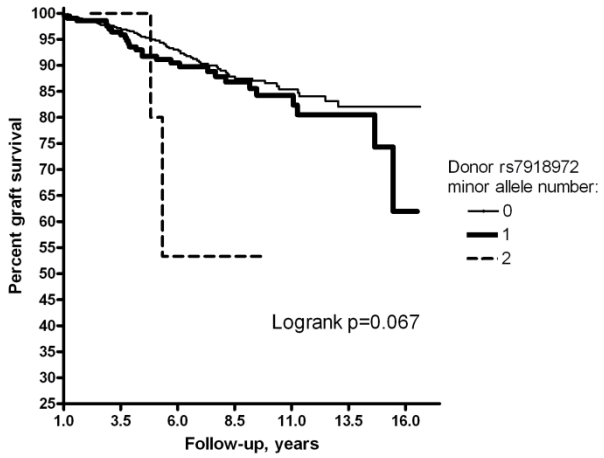

Figure 2. Curves of long-term renal graft survival by donor rs7918972 genotype.

Numbers 0 to 2 designate corresponding number of the minor allele copies per genotype. The logrank test showed borderline statistical significance of the differences between the respective curves.

Table 4. *CUBN* SNPs in the longitudinal study with follow-up for graft failure.

Genotype	*CUBN* SNPs		**Graft failure**, n=92	**Functioning graft**, n=870	HR [95% CI][a]	*p* value[a]	HR [95% CI][b]	*p* value[b]
Donor	**rs7918972**	Genotypes, count MAF, %	2/26/64 **16.3%**	8/171/691 **10.7%**	**1.50 [0.99-2.26]**	**0.056**	**1.53 [0.99-2.37]**	**0.055**
	rs1801239	Genotypes, count MAF, %	2/15/75 10.3%	11/201/658 12.8%	0.80 [0.49-1.30]	0.363	0.75 [0.43-1.31]	0.311
Recipient	rs7918972	Genotypes, count MAF, %	3/22/67 15.2%	15/234/621 15.2%	0.94 [0.62-1.43]	0.773	1.00 [0.64-1.56]	0.992
	rs1801239	Genotypes, count MAF, %	0/20/72 10.9%	6/212/651 12.9%	0.78 [0.48-1.26]	0.308	0.70 [0.40-1.25]	0.229

HR, hazard ratio; CI, confidence interval.
[a] univariate Cox regression
[b] multivariate Cox regression model adjusted for donor and recipient age and sex, donor type (living or deceased), ischemia times, immunosuppressive medication use, and history of acute rejection episodes.

We found donor rs7918972 to be associated with proteinuria levels cross-sectionally at 1 year of post-transplant follow-up (beta 0.201, p=0.015) [**Table 5**]. No association between the SNPs and renal function by measured GFR or creatinine clearance was observed at the same time-point; however, donor rs7918972 showed a directionally consistent, although not statistically significant, trend for association with an increased rate of GFR decline (data not shown).

Table 5. *CUBN* SNPs association with urinary total protein excretion cross-sectionally at 1 year after transplantation.

Genotype	SNP	Univariate Tobit regression			Multivariate Tobit regression[a]		
		Coefficient	SE	*p* value	Coefficient	SE	*p* value
Donor	**rs7918972**	**0.223**	**0.083**	**0.007**	**0.201**	**0.082**	**0.015**
	rs1801239	-0.039	0.078	0.617	-0.021	0.077	0.784
Recipient	rs7918972	-0.072	0.071	0.313	-0.049	0.070	0.488
	rs1801239	-0.028	0.081	0.726	-0.023	0.080	0.779

[a]Model adjusted for donor and recipient age and sex, donor type (living or deceased), systolic and diastolic blood pressure. Coefficients are given per copy of the minor allele.

Discussion

In the present study we followed up the results of a recent GWAS, which identified the cubilin gene *CUBN* as a locus for albuminuria [4]. As albuminuria is an established risk factor for progressive renal function loss, the GWAS findings raised the hypothesis that genetic variation in the *CUBN* locus could be associated with progressive renal function loss and finally end stage renal disease. To test this hypothesis we studied the cited top SNP as well as a tagSNP in *CUBN* in relation to final renal clinical outcomes, namely ESRD in native kidneys and GF in the transplanted kidney.

In a case-control design we studied rs7918972 and rs1801239 genotypes in ESRD patients versus kidney donors. The MAF for rs7918972 was significantly higher in ESRD patients as compared to kidney donors, imposing a 39% increased risk for ESRD per copy of the minor allele. Follow-up data after transplantation showed direction-consistent trend for an association between donor kidney rs7918972 and development of GF in recipients. Thus, the SNP in *CUBN* locus was associated with susceptibility to develop ESRD in two settings, namely ESRD in native kidneys and GF in transplanted kidneys.

Transplantation represents a unique setting, also from genetic point view: an organ with its own genotype functions in an organism with another genotype. We tested both donor and recipient genotype for association with the renal outcome to investigate whether it is the kidney genotype that determines its own fate or it is the recipient genotype that

influences function and survival of the transplanted organ. This unique design is useful for genetic research in nephrology as it enables discrimination between the renal and extra-renal mechanisms [12].

In our study, it was donor rather than recipient *CUBN* genotype that was associated with GF, suggesting involvement of local, intra-renal pathways in processes of transplanted kidney survival which are independent of systemic influences.

Albuminuria is known as a predictor of cardiovascular and non-cardiovascular mortality [13]. However, in our study *CUBN* genotypes did not associate with cerebro- or cardiovascular accident as a cause of death in donors and cardiovascular and all-cause mortality after transplantation in recipients.

As no albuminuria data were available and urinary albumin levels are known to correlate with total protein, we tested association of the *CUBN* SNPs with 24-h total urinary protein excretion as a surrogate phenotype. Interestingly, we found donor rs7918972 to be associated with elevated proteinuria levels cross-sectionally at 1 year after transplantation. This is consistent with our results of association with the outcome, and also in line with the results of a recent study which revealed, using exome sequencing, a deleterious mutation in *CUBN* in a family of proteinuric patients, thus confirming the *CUBN* gene involvement in proteinuria [14].

In the original GWAS [4] the *CUBN* SNP rs1801239, associated with elevated urinary albumine-to-creatinine ratio and microalbuminuria. However, this SNP was not associated with CKD or estimated GFR. In agreement with this, our case-control study showed no association between this SNP and ESRD. Also, rs1801239 was not associated with GF in our longitudinal study Instead, it was the other *CUBN* polymorphism, the tagSNP rs7918972, that was associated with ESRD in our study.

The *CUBN* locus is characterized by a high variability, with both common and rare mutations. Mutations in the *CUBN* locus are known to be the cause of Imerslund-Gräsbeck syndrome (OMIM #261100, Finnish type) which is a rare (the estimated prevalence is <6:1,000,000) autosomal recessive disorder characterized by vitamin B_{12} deficiency commonly resulting in megaloblastic anemia, and also neurological damage and mild proteinuria [15]. However, we did not aim to address previously clinically-associated Mendelian mutations in the *CUBN* in our study. We aimed to investigate whether common variation, as opposed to rare mutations in Imerslund-Gräsbeck syndrome, in the *CUBN* associates with kidney disease. In the same time, we targeted a lower part of the common variability range, with MAFs between 10 and 15%, aiming to reveal allegedly stronger genetic effects. We selected two SNPs in the *CUBN* locus for the present study: first, the one previously published to be associated with albuminuria levels in the general population, i.e. the missense variant (Ile2984Val) rs1801239, and second the tagSNP in the *CUBN* intron, rs7918972. The latter is in high linkage disequilibrium (r^2=0.831) with another missense variant rs1801232 (Asn3552Lys) in *CUBN*, which might be responsible for the biological impact of the polymorphism on the

protein level. The minor allele of rs1801232 leads to an asparagine-to-lysine amino acid substitution in the C-terminal CUB27 domain of cubilin. Despite the amino acids differ in chemical properties (isoelectric points: Asn 5.4, Lys 9.8), the substitution was predicted to be benign by bioinformatics algorithms. However, the mutation is close to sites of N-glycosylation (amino acid 3533) and di-sulfide bond (between amino acids 3564 and 3586) and therefore might potentially interfere with secondary protein structure and, consequently, function. The Imerslund-Gräsbeck syndrome mutations, for which functionality was proven, affect the IF-cobalamin-binding region in the CUB8 domain of cubilin (rs121434430 Pro1297Leu), CUB6 domain (*CUBN* IVS6 C-G in-frame insertion) or CUB23 domain (*CUBN* IVS23 G-T transversion at the conserved donor splice site of exon 23). The SNPs that we studied were spatially distant from these variants and located to the CUB22 domain (rs1801239) and CUB27 domain (rs1801232 tagged by rs7918972).

The rs7918972 *CUBN* SNP, associated with ESRD and GF in our study, is localized in high proximity to the neighboring gene, *RSU1*. Although the nine SNPs tagged by rs7918972 are all located in the *CUBN* locus, linkage disequilibrium with and involvement of the *RSU1* is theoretically possible and cannot be entirely ruled out (**Suppl. Fig S1**). The *RSU1* gene encodes Ras suppressor protein 1, which participates in the Ras signal transduction pathway, growth inhibition and nerve-growth factor induced differentiation processes. Its mRNA is expressed in the kidney (according to the NCBI GEO profiles), in a low-to-moderate quantity (51 transcripts per million, according to the NCBI EST profiles). However, functional proof is beyond the scope of the present study, and further research will be needed to discriminate between the effects of these neighboring genes.

Interestingly, we found an interaction between the two SNPs studied. According to our data, the rs7918972 minor allele requires a copy of the rs1801239 minor allele to express its risk phenotype, whereas the minor allele of rs1801239 displays protective effect in the absence of rs7918972 minor allele. This pattern was observed in the case-control study and warrants further investigation to determine whether found statistical interaction has biological implications.

Our study was conducted in kidney transplant recipients, and thus reflects a population that developed ESRD in their native kidneys and was eligible to receive a transplantation. As such, renal transplant recipients represent a relatively healthy subset of the ESRD population, a selection bias inherent to any study in renal transplantation. This should be considered a limitation to our study. In chronic renal disease, both in native kidney disease and in transplantation, mortality is high, and for analyses on ESRD the competing risks of mortality, in particular cardiovascular, are therefore relevant to consider. In the current population, mortality with functioning graft was 15.8 percent, and no association with either of the *CUBN* SNPs was observed.

Our longitudinal study of graft failure may have been underpowered to detect a significant SNP effect. Insufficient power might thus be an explanation of the fact that

convincing statistical significance was not reached in the graft survival analysis for association with rs7918972. Studies in larger populations are warranted to confirm an association between the *CUBN* SNP and GF.

Conclusion

Our study confirms association of the *CUBN* with renal phenotypes of progressive renal function loss and urine protein loss. We first identified *CUBN* SNP rs7918972 as a novel genetic variant of susceptibility for ESRD in a case-control design. In a separate proof-of-principle longitudinal study, which served as an internal replication, we reproduced the association. Thus, rs7918972 was associated with susceptibility to develop progressive renal function loss in two settings, namely ESRD in native kidneys and GF in transplanted kidneys. It was kidney genotype that associated with increased risk, supporting impact of intra-renal pathways on organ damage. Our study set-up – analyzing both donor and recipient genotypes – provides a powerful design for hypothesis-driven studies on risk loci for renal damage enabling differentiation between local, intra-renal, and systemic, extra-renal, influences.

Supplementary Material

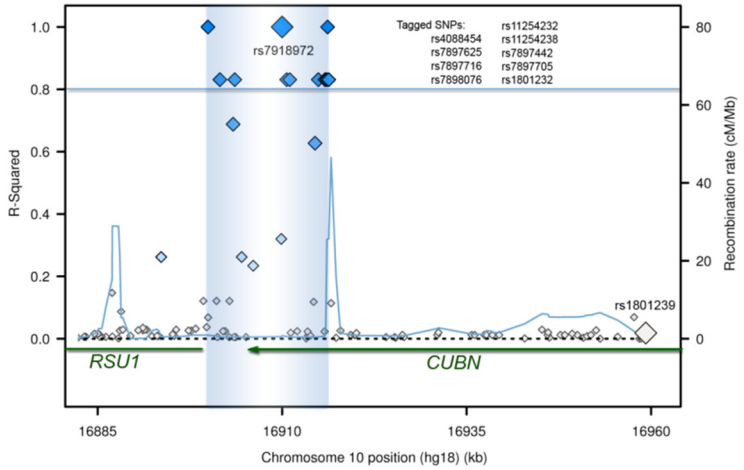

Figure S1. *CUBN* regional LD plot.

The figure was generated using HapMap data (release 22, CEU population). The horizontal blue line represents an arbitrarily chosen LD threshold (r^2=0.8). SNPs are shown as diamonds. The color gradient between the diamonds reflects the pairwise LD between the SNPs, with color intensity of each diamond being directly proportional to the r^2 value. Boundaries of the gene coding regions are shown as green horizontal lines. The largest size diamonds represent the present study SNPs. The shaded area designates a span of the gene region tagged by rs7918972.

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Chapter 6

SLC22A2 is associated with tubular creatinine secretion and bias of estimated GFR in renal transplantation

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Abstract

Genome-wide association studies reported *SLC22A2* variants to be associated with serum creatinine. As *SLC22A2* encodes the organic cation transporter 2 (OCT2), the association might be due to an effect on tubular creatinine handling. To test this hypothesis we studied the association of *SLC22A2* polymorphisms with phenotypes of net tubular creatinine secretion: fractional creatinine excretion (FE_{creat}) and bias of estimated glomerular filtration rate (eGFR). We also studied the association with end-stage renal disease (ESRD) and graft failure (GF) in renal transplant recipients.

SLC22A2 SNPs, rs3127573 and rs316009, were genotyped in 1142 ESRD patients receiving renal transplantation and 1186 kidney donors as controls. GFR was measured with ^{125}I-iothalamate clearance. Creatinine clearance was also assessed. FE_{creat} was calculated from the simultaneous clearances of creatinine and ^{125}I-iothalamate.

Donor rs316009 was associated with FE_{creat} (beta -0.053, p=0.024), and with estimated (MDRD and CKD-EPI) but not measured GFR. In line, donor rs316009 was associated with bias of the MDRD and CKD-EPI but not the Cockroft-Gault equation. Both SNPs were associated with ESRD: odds ratios [95% CI] 1.39 [1.16-1.67], p=0.00065, and 1.23 [1.02-1.48], p=0.042, for rs3127573 and rs316009, respectively. Neither SNP was associated with GF.

Thus, *SLC22A2* is associated with phenotypes of net tubular creatinine secretion and ESRD..

Introduction

Genome-wide association studies (GWAS) identified a number of loci influencing renal function (7, 23, 24) usually using renal function estimates based on serum creatinine as a proxy for glomerular filtration rate (GFR).

Serum creatinine, however, is not only determined by GFR, but also by the rate of creatinine generation, extrarenal elimination and by tubular handling (5, 31, 35, 46, 49). Dissecting the biological mechanisms underlying genetic associations with serum creatinine, therefore, is pivotal for proper interpretation of GWAS findings. In particular, it is relevant to assess whether loci associated with serum creatinine reflect susceptibility to renal damage (1), or, alternatively, reflect biological variations in creatinine generation or in creatinine handling unrelated to the risk for renal damage.

The *SLC22A2* gene is associated with serum creatinine and estimated GFR (eGFR) (7). However, *SLC22A2* might well be a creatinine secretion locus. First, *SLC22A2* SNPs were associated with GFR estimated from serum creatinine but not cystatin C (23, 34). Second, the *SLC22A2* gene encodes the organic cation transporter 2, OCT2, a predominant transporter involved in tubular creatinine secretion. It is expressed in the basolateral membrane of proximal tubule cells (29), where it mediates creatinine uptake from the peritubular capillaries, as an initial step in tubular creatinine secretion (9, 29, 38, 51, 52, 59).

Tubular secretion accounts for 10-20% of urinary creatinine excretion (5, 35, 46). Presence of tubular secretion as an additional mechanism of creatinine clearance translates into an acknowledged systematic error (bias) for creatinine-based GFR estimation methods (35, 45, 46, 49).

In the present study we test the hypothesis that the association between genetic variation in the *SLC22A2* locus and eGFR is due to association of *SLC22A2* with tubular secretion of creatinine. To this purpose, we investigated first the association of *SLC22A2* variants with fractional excretion of creatinine (FE_{creat}) as an intermediate phenotype for tubular creatinine secretion. Second, we tested the association of *SLC22A2* with the bias of eGFR assessed by the Modification of Diet in Renal Disease (MDRD) and the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equations. As a "negative control" we also tested the association of *SLC22A2* with the bias of the creatinine clearance estimated by the Cockroft-Gault equation, hypothesizing that *SLC22A2* would be associated with bias of the MDRD and CKD-EPI equation, but not with that of the Cockroft-Gault equation as the latter is calibrated on creatinine clearance. Finally, we tested the association of *SLC22A2* with end-stage renal damage in native and transplanted kidneys.

The studies were performed in the REGaTTA (REnal GeneTics TrAnsplantation, Groningen, the Netherlands) cohort (12, 40), a large single-center renal transplant cohort, where we genotyped both donors and recipients. Since the *SLC22A2* gene

expression is restricted to the kidney with scarce extra-renal sites of expression (placenta, brain) [NCBI UniGene database (58)], donor *SLC22A2* genotype largely represents kidney genotype; accordingly, we tested associations between the recipient phenotypes and the donor *SLC22A2* polymorphisms. For this study we selected the two most prominent among previously characterized *SLC22A2* polymorphisms: rs3127573 and rs316019. The first SNP, rs3127573 in the *SLC22A2* 5′ region, was the top SNP reported for association with serum creatinine and eGFRcreat (7). The other, a missense rs316019, is the most frequent coding variant in the *SLC22A2* gene and is also the best functionally characterized. This polymorphism in the exon 4 results in an Ala270Ser amino acid substitution with functional consequences for the protein and was associated with impaired transport of OCT2 substrates in vitro (8, 15, 48, 57, 61). Because genotyping of rs316019 was technically not possible in this study, we searched for another SNP to use as a proxy. Thus, rs316009, a highly correlated tag SNP in perfect linkage disequilibrium (LD: r^2=1) in the *SLC22A2* intron, was genotyped instead, assuming that it represents rs316019. Therefore, in the subsequent manuscript sections we refer to rs3127573 and rs316009.

Methods & Materials

Study population

This study was conducted in the REGaTTA cohort, with assessment of association with end-stage renal disease (ESRD) in the total cohort, and assessment of association with the intermediate phenotypes in distinct sub-groups where the detailed phenotypes were available. The selection flowchart of the study is shown in **Figure 1**. Patient characteristics, transplantation-related parameters, clinical and laboratory data were retrieved from medical records.

1) The total cohort consisted of 1184 kidney donors and 1141 first graft recipients (1066 matched donor-recipient pairs), assessed post-hoc from all renal transplantations in our center between 1993 and 2008, excluding cases of re-transplantation, combined kidney/pancreas or kidney/liver transplantation, technical problems, absence of DNA and loss of follow-up. After transplantation the recipients were followed up during median [IQR] 5.5 [3.0-8.7] years for graft failure (GF) defined as return to dialysis or re-transplantation, censored for death with a functioning graft. Allele frequency in recipients versus donors was analyzed in a case-control approach to test for the association of *SLC22A2* with ESRD. Follow-up data in the recipients were used for analysis of association with GF.

2) Data on ^{125}I-iothalamate clearance at 1 year after transplantation, serum creatinine and creatinine clearance from 24 hour urine, collected the day before ^{125}I-iothalamate

clearance measurements, were available for 738 recipients. These data were used to assess the association of *SLC22A2* with the bias of eGFR.

3) Simultaneously measured 2-hour clearances of creatinine and ^{125}I-iothalamate were available in 348 recipients, studied at median [IQR] 3.5 [1.7-6.9] years after transplantation. These data were used to calculate FE_{creat} and analyze for its association with *SLC22A2*. The overlap between the subgroups of patients with true GFR and FEcreat measurements (n=738 and n=348, respectively) was 264 cases.

The Institutional Review Board of the University Medical Center Groningen approved the study protocol. Written informed consent was given by all recipients and living donors. For deceased donors, with research carried out after the organ removal and implantation, no consent was required by Dutch law. The study was conducted according to the principles of the Declaration of Helsinki. All the data were anonymized prior to analyses.

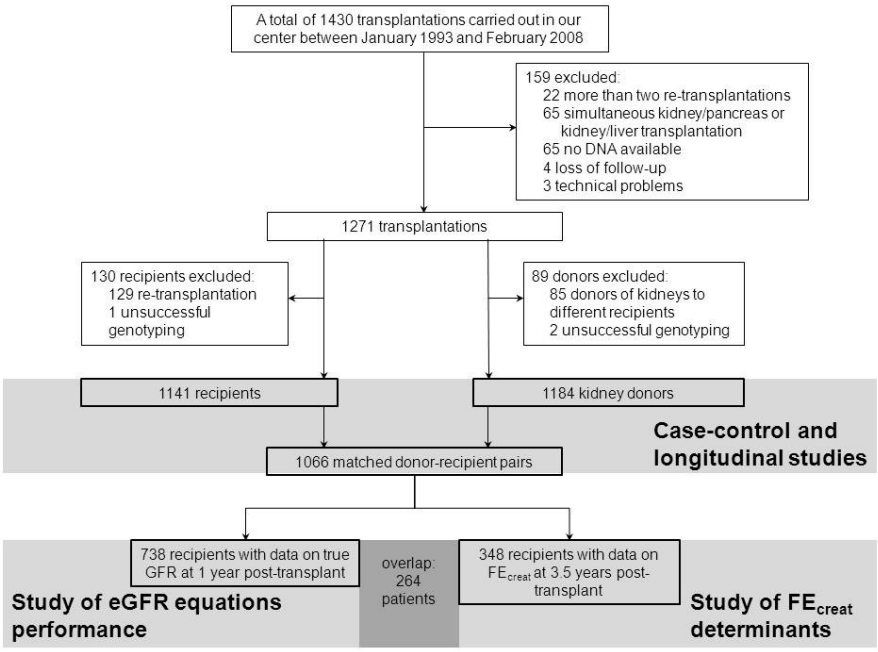

Figure 1. A flowchart of the study participants selection.

All cases of renal transplantation carried out in the University Medical Center Groningen in 1993-2008 were considered for inclusion. The exclusion criteria were re-transplantation, combined kidney/other organ transplantation, technical problems, absence of DNA and loss of follow-up. A total of 1141 end-stage renal disease patients (recipients) and 1184 kidney donors were successfully genotyped and included into the subsequent analyses. The grey-shaded areas indicate different studies performed to answer corresponding research questions of the present project.

(e)GFR, (estimated) glomerular filtration rate; FEcreat, fractional excretion of creatinine.

Treatment protocols and renal function measurement

The treatment regimen in our transplant population has been described in more detail elsewhere (13). Briefly, standard immunosuppression consisted of cyclosporine micro emulsion (Neoral, Novartis, Pharma b.v., Arnhem, the Netherlands; 10 mg/kg; trough-levels idem) and prednisolone from March 1993 until May 1996, mycophenolate mofetil (Cellcept, Roche, Nederland b.v., Woerden, the Netherlands; 2 g/d) was added from May 1997 to date.

The routine follow-up includes measurement of true GFR at regular intervals by 125I-iothalamate clearance, as described in detail by Visser *et al.* (55). Creatinine clearance was also measured as a routine, from 24-hour urine collected on the day preceding the GFR assessment. As of 2006 simultaneous measurement of creatinine clearance was included in the protocol, as described in detail by Sinkeler *et al.* (46). Briefly, serum and 2-h urine creatinine measurements were obtained during the measurement of 125I-iothalamate GFR and FE_{creat} was calculated as $(U/P)_{creat}/(U/P)_{iot}$ from the same samples as the ^{125}I-iothalamate clearance, thus avoiding discrepancies due to collection errors and diurnal changes in renal function. Creatinine was measured with the Roche enzymatic creatinine assay, which is isotope dilution mass spectrometry-traceable. The assay was calibrated to the reference standard (Cleveland Clinic Laboratory measurements), as proposed by Coresh *et al.* (11), and described in more detail by Tent *et al.* (50).

DNA isolation and genotyping

DNA was extracted from peripheral whole blood (in recipients and living donors) or lymph nodes/spleen lymphocytes (in deceased donors) using a commercial kit following the manufacturer's instructions, transferred into 2 ml Eppendorf tubes and stored at -20°C. Absorbance at 260 nm was measured with NanoDrop spectrophotometer (ND-1000, NanoDrop Technologies) and DNA concentration was calculated by the NanoDrop nucleic acid application module. As a measure of DNA purity 260/280 and 260/230 absorbance ratios were assessed. Where samples failed to meet the minimum DNA concentration and purity recommended for Illumina genotyping, repeated isolation attempts were made.

Genotyping of rs316019 was not possible for technical reasons as the polymorphism was not supported by the used assay. Instead we genotyped rs316009, an intronic *SLC22A2* SNP which is in perfect linkage disequilibrium (r^2=1.0) with rs316019. Genotyping of the selected SNPs, rs3127573 and rs316009, was performed using the Illumina VeraCode GoldenGate assay kit (Illumina, San Diego, CA, USA), according to the manufacturer's instructions. Genotype clustering and calling were performed using BeadStudio Software (Illumina).

Statistical analysis

Analyses were performed with PASW Statistics 18.0 (SPSS Inc., Chicago, IL) and PLINK v1.07 (S. Purcell, http://pngu.mgh.harvard.edu/purcell/plink/ (37)).

Genotype-phenotype associations were tested under an additive genetic model and results (regression coefficients and p-values) are reported per copy of the minor allele.

Association between the *SLC22A2* SNPs and FE_{creat} was studied with linear regression. Relevant covariates (age, sex, BMI, drugs use) were included in the multivariate model. Drugs use was coded as yes/no co-trimoxazole and cyclosporin A/tacrolimus/no calcineurin inhibitor.

MDRD (25, 26), CKD-EPI (27) and Cockroft-Gault (10) equations were used to calculate eGFR. Predictive performance of the equations for GFR estimation was analyzed by assessing their precision (the scatter of the observations and expressed as R^2), 30 and 10% accuracy (the percentage of subjects within, respectively, 30 and 10% of true GFR, i.e. ^{125}I-iothalamate clearance) and bias (the mean prediction error calculated as ∑(predicted value - true value)/n) (2).

Statistical significance of differences in renal function, its estimates and bias of GFR estimation between the *SLC22A2* SNPs genotypes was tested with Kruskal-Wallis test (similarly to the general genotypic 2df-test).

Association with ESRD was tested in a case-control design by comparing 1141 ESRD patients admitted for renal transplantation and 1184 kidney donors. The PLINK DFAM algorithm was used to account for case-control relatedness within living-donor transplantation cases.

The effect of the SNPs on graft survival was studied in 1066 renal transplant recipients. A Cox regression analysis was performed including known predictors of GF (donor and recipient age and sex, donor type, ischemia times, delayed graft function and acute rejection episodes history, immunosuppressive drugs) as covariates in a multivariate model. Kaplan-Meier survival curves were built for analysis and visualization of graft survival in genotypic groups, and the log rank test was used to test for significance of differences between the curves.

Results

Main patient characteristics are presented in Table 1 for the total cohort and for the subgroups.

In the total cohort, the minor allele frequencies were 11.6% for rs316009 and 13.2% for rs3127573. There was no deviation from Hardy-Weinberg equilibrium in controls – donors (p=0.47 and p=1.00 for rs316009 and rs3127573, respectively). There was no linkage disequilibrium between the two SNPs (r^2=0.002 in our cohort, r^2=0.008 in HapMap release 22 CEU population).

Table 1. Patients characteristics:

1A. Baseline parameters at transplantation and outcomes during 5.5 [3.0-8.7] years of follow-up in the total cohort

1066 matched donor-recipient pairs	
Recipient age (years), mean±SD	48.1±13.5
Recipient sex: male, n (%)	621 (58.3)
Donor age (years), mean±SD	44.6±14.3
Donor sex: male, n (%)	540 (50.7)
Living donors, n (%) - from which related donors, n (%)	260 (24.4) 149 (14.0)
Cold ischemia time (hours), mean±SD	16.4±9.7
Delayed graft function, n (%)	331 (31.1)
Acute organ rejection episodes history, n (%)	369 (34.6)
Death-censored graft failure, n (%)	172 (16.1)
Death with a functioning graft, n (%)	183 (17.2)

1B. Subgroups of patients with refined phenotype of renal function

	Recipients at 1 year post-transplant	Recipients at 3.5 years post-transplant
N	738	348
Recipient age (years), mean±SD	48.5±13.7	53.7±13.1
Recipient sex: male, n (%)	431 (58.4)	182 (52.3)
Serum creatinine (µmol/l), mean±SD	144.6±49.5	128.1±51.1
Creatinine clearance (ml/min), mean±SD	63.3±21.9	70.5±23.0
Measured GFR (ml/min), mean±SD	55.1±19.1	56.2±20.8
FE_{creat}, mean±SD	-	1.05±0.18
Recipient BMI (kg/m^2), mean±SD	26.4±4.3	26.6±4.7
Co-trimoxazole use, n (%)	-	19 (5.5)
Calcineurin inhibitor type: cyclosporin A, n (%)	531 (72)	189 (54.3)

Association of the SLC22A2 variants with FE_{creat}, renal function estimates and performance of eGFR

In 348 recipients FE_{creat} was assessed from simultaneous clearances of creatinine and ^{125}I-iothalamate. It ranged from 0.71 (min) to 1.39 (max) [**Figure 2**], with a mean of 1.05, indicating a 5% mean contribution of tubular secretion to creatinine clearance. Determinants of FE_{creat} are given in **Table 2** displaying univariate effects of donor and recipient characteristics and the multivariate linear regression model. The donor kidney rs316009 (the proxy SNP for the functional rs316019) was associated with lower FE_{creat} in an allele dose-dependent manner (beta -0.053 per copy of the minor allele, p=0.024) in the model which included donor age and sex, recipient age, sex, BMI, use of co-trimoxazole and type of calcineurin inhibitor.

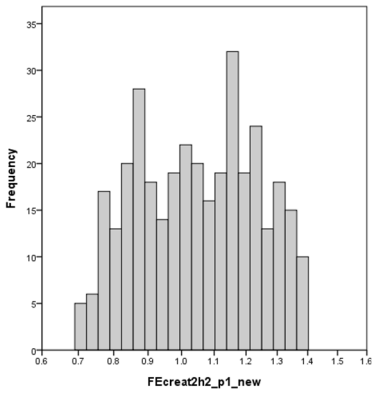

Figure 2. A histogram of the fractional creatinine excretion (FE_{creat}) values in 348 renal transplant recipients at median [IQR] 3.5 [1.7-6.9] years after transplantation.

The distribution of FE_{creat} values is demonstrated, as well as the fact that in a proportion of the recipients FE_{creat} is below 1.

Table 2. Determinants of fractional creatinine excretion in 348 renal transplant recipients at median [IQR] 3.5 [1.7-6.9] years after transplantation.

	Univariate			Multivariate		
	Beta	SE	p value	Beta	SE	p value
Donor rs316009	-0.038	0.021	0.078	-0.053	0.024	**0.024**
Donor rs3127573	0.035	0.023	0.119	0.021	0.027	0.433
Donor age	-0.0004	0.001	0.550	1.6×10^{-4}	0.001	0.725
Donor sex: male	-0.016	0.02	0.426	0.05	0.024	**0.039**
Recipient age	-0.003	0.001	**<0.001**	-0.005	0.001	**<0.001**
Recipient sex: male	0.175	0.017	**<0.001**	0.179	0.024	**<0.001**
Recipient BMI	0.007	0.003	**0.016**	0.009	0.003	**0.001**
Co-trimoxazole use	0.009	0.043	0.839	0.129	0.156	0.391
Calcineurin inhibitor type	-0.004	0.011	0.686	-0.028	0.013	**0.035**

Regression coefficients are presented per copy of the minor allele. SE, standard error.

Association analyses of renal function, its estimates and performance of eGFR were conducted in 738 recipients at 1 year post-transplantation. Precision (R^2) of the GFR estimation methods was 0.59, 0.60 and 0.48, for MDRD, CKD-EPI and Cockcroft-Gault formulas, respectively. 10% and 30% accuracy were 23.8% and 74.3%, 29.8% and 81.8%, 29.5% and 76.0% for MDRD, CKD-EPI and Cockcroft-Gault equations, respectively.

Data on renal function and renal function estimates are given in **Table 3** by a breakup by donor kidney genotype. There was no difference in serum creatinine or creatinine clearance between the genotypes. Measured GFR was not different between the genotypes either. However, MDRD and CKD-EPI eGFR was slightly but significantly higher with each copy of the rs316009 minor allele in the kidney genotype (p=0.016 and p=0.022 for MDRD and CKD-EPI, respectively). This was paralleled by a decrease in GFR estimation bias of MDRD and CKD-EPI equations across the rs316009 genotypes, with eGFR closely approximating actual GFR in the minor allele homozygotes. Cockcroft-Gault equation performance was not affected by the genotypes. The rs3127573 was not significantly associated with renal function estimates.

Association of the SLC22A2 variants with phenotypes of renal function loss – ESRD and GF

The genotypes of the studied *SLC22A2* variants were unequally distributed between the ESRD patients and kidney donors. There were more carriers of both SNPs minor alleles among ESRD patients than among donors. Correspondingly, the MAFs of rs3127573 and rs316009 were significantly higher in ESRD patients (14.9% and 12.9%, respectively) as compared to kidney donors (11.4% and 10.6%, respectively), implicating an increased risk of ESRD in association with the minor allele. In the additive genetic model adjusted for age, sex and case-control relatedness odds ratios (OR) [95% CI] for ESRD were 1.39 [1.16-1.67] per copy of the rs3127573 minor allele, p=0.00065, and 1.23 [1.02-1.48] per copy of the rs316009 minor allele, p=0.042 (**Table 4**).

During follow-up of a median [IQR] 5.5 [3.0-8.7] years 172 (16.1%) cases of GF occurred and 183 (17.2%) patients died with a functioning graft. In a multivariate Cox regression model adjusted for donor and recipient age and sex, donor type, cold ischemia duration, history of delayed graft function and acute rejection episodes, immunosuppressive drugs none of the two donor kidney *SLC22A2* SNPs was significantly associated with GF under an additive genetic model (**Table 5**). Log rank test showed p=0.530 and p=0.078 for significance of differences between the graft survival curves by donor rs316009 and rs3127573 genotypes, respectively. None of the SNPs was associated with overall or cardiovascular mortality in recipients (not shown).

Table 3. Renal function and its creatinine-based estimates in 738 renal transplant recipients at 1 year after transplantation, subdivided by genotypes of the donor kidney *SLC22A2* variants.

Renal function parameters	Donor rs316009			*p* value
	0	1	2	
	n=594	n=135	n=9	
Serum creatinine (µmol/l)	135.0 (112.0-171.8)	127.5 (106.0-167.3)	134.0 (120.5-142.5)	0.055
Creatinine clearance (ml/min)	61.0 (48.0-76.0)	64.0 (46.5-79.3)	62.0 (48.0-66.0)	0.797
Measured GFR (ml/min)	54.0 (42.0-68.0)	53.0 (42.0-65.3)	55.0 (40.5-58.0)	0.756
eGFR MDRD (ml/min)	43.1 (34.7-53.7)	43.9 (36.6-57.4)	51.4 (49.0-53.7)	**0.016**
eGFR CKD-EPI (ml/min)	46.6 (36.8-58.0)	49.2 (38.8-64.3)	55.3 (53.1-58.0)	**0.022**
eGFR Cockcroft-Gault (ml/min)	59.6 (48.2-73.8)	65.4 (49.0-76.2)	64.2 (56.8-71.5)	0.307
Bias MDRD (ml/min)	-10.5 (-17.5- -3.0)	-6.8 (-15.0-1.0)	-1.9 (-6.1-7.1)	**0.001**
Bias CKD-EPI (ml/min)	-6.9 (-13.6-0.5)	-2.9 (-11.6-5.1)	2.2 (-2.1-9.1)	**<0.001**
Bias Cockcroft-Gault (ml/min)	-0.8 (-11.3-8.1)	1.3 (-7.9-10.6)	6.3 (-4.6-10.4)	0.257

Renal function parameters	Donor rs3127573			*p* value
	0	1	2	
	n=579	n=151	n=7	
Serum creatinine (µmol/l)	134.0 (110.5-170.5)	136.0 (114.0-172.0)	115.0 (100.8-162.0)	0.595
Creatinine clearance (ml/min)	60.0 (47.0-76.0)	64.0 (49.0-77.0)	73.5 (69.8-97.5)	0.076
Measured GFR (ml/min)	54.0 (41.0-67.0)	54.0 (44.0-67.0)	60.0 (56.3-74.3)	0.565
eGFR by MDRD (ml/min)	43.1 (34.7-54.4)	43.9 (35.2-53.6)	46.9 (35.0-51.2)	0.884
eGFR CKD-EPI (ml/min)	47.1 (37.0-59.5)	47.6 (37.9-57.1)	48,4 (32,8-52,3)	0.829
eGFR Cockcroft-Gault (ml/min)	60.5 (48.2-74.2)	60.2 (49.6-74.0)	55,4 (47,8-73,5)	0.901
Bias MDRD (ml/min)	-9.0 (-16.6- -1.9)	-10.2 (-18.4- -4.1)	-20.4 (-27.9- -10.6)	0.104
Bias CKD-EPI (ml/min)	-5.7 (-12.8-1.6)	-7.5 (-14.8- -0.4)	-9,5 (-23,1- -7,2)	0.058
Bias Cockcroft-Gault (ml/min)	-0.2 (-10.3-9.0)	0.3 (-12.1-7.5)	-21,0 (-42,4-1,5)	0.110

Data are presented as medians (IQR). Numbers 0 to 2 designate corresponding number of the minor allele copies per genotype. Statistical significance of the differences was tested with Kruskal-Wallis test.

Table 4. *SLC22A2* SNPs in the case-control study of ESRD patients versus kidney donors: genotypes distributions and minor allele frequencies

SLC22A2 SNPs		ESRD patients, n=1141	Kidney donors, n=1184	OR [95% CI] per copy of the minor allele	*P* value [a]
rs3127573	Genotypes, count MAF, %	11 / 317 / 813 14.9	12 / 245 / 927 11.4	1.39 [1.16-1.67]	6.54×10^{-4}
rs316009	Genotypes, count MAF, %	14 / 260 / 867 12.6	13 / 224 / 947 10.6	1.23 [1.02-1.48]	0.04

MAF, minor allele frequency; OR, odds ratio; CI, confidence interval.

[a] Additive test with adjustment for age, sex and case-control relatedness

Table 5. Donor kidney *SLC22A2* SNPs association with incidence of graft failure in renal transplant recipients

SLC22A2 SNPs	Univariate Cox regression HR [95% CI]	*P* value	Multivariate Cox regression HR [95% CI][a]	*P* value[a]
rs3127573	1.21 [0.88-1.65]	0.244	1.16 [0.85-1.59]	0.343
rs316009	1.20 [0.87-1.65]	0.269	1.26 [0.90-1.75]	0.175

HR, hazard ratio; CI, confidence interval.

Cox regression analyses were run under the additive genetic model, the coefficients are presented per copy of the minor allele.

[a] Adjusted for donor and recipient age and sex, donor type (living vs deceased), cold ischemia duration, history of delayed graft function and acute rejection episodes, immunosuppression drugs

Discussion

This study in renal transplant recipients reports a significant association between donor *SLC22A2* SNP rs316009 and FE_{creat}, with lower FE_{creat} in the presence of one or two minor alleles. An association was found between rs316009 and eGFR (MDRD and CKD-EPI), but not with measured GFR. In line, bias of the eGFR equations was significantly associated with rs316009. Remarkably, both *SLC22A2* SNPs were associated with the increased risk for ESRD in native kidneys. Thus, genetic variation in *SLC22A2* can modulate net tubular creatinine handling, hence affecting the bias of the main renal function equations that are calibrated on measured GFR. The association with ESRD is in line with a role for tubular functions in progressive renal function loss, and deserves further substantiation.

By the required sample size, GWA studies for renal function usually rely on simple renal function estimates, mostly serum creatinine and $eGFR_{creat}$ (7), sometimes complemented by renal function estimates with cystatin C (23, 24), to account for the short-comings of serum creatinine and/or eGFR for renal function assessment. Dissociation in outcomes for association with creatinine and cystatin C (34) is useful to separate genetic loci that are related to true GFR from loci that affect creatinine level by creatinine generation or tubular secretion. Thus, the *SLC22A2* locus emerges as a locus likely to influence creatinine secretion rather than true GFR (24), as supported by the current study.

Tubular secretion of creatinine has wide inter-individual variability (35, 45). We show that genetic variation in the *SLC22A2* locus, i.e. the *SLC22A2* SNP rs316009, in the donor kidney is an independent determinant of FE_{creat}. The lower FE_{creat} per copy of the rs316009 minor allele is consistent with prior in vitro studies showing that the correlated polymorphism, rs316019, which we assumed to be the true causal variant tagged by rs316009, resulted in decreased transport of OCT2 substrates (8, 15, 48, 57, 61).

In line with data in healthy subjects, FE_{creat} was associated with age, gender and BMI, supporting the robustness of this phenotype and its determinants (46). Our current data, for the first time, demonstrate that genetic factors are also involved in FE_{creat}. Age, sex and BMI are all related to muscle mass and hence creatinine supply, a possible common denominator in the association with tubular creatinine secretion. Of note, these factors can influence creatinine transporter expression. In rodents, pronounced sexual dimorphism in renal *SLC22A2* expression was reported, with higher mRNA levels (47) and greater transport of OCT2 substrates in renal slices from male rats (53). In line, testosterone up-regulates and estradiol moderately down-regulates rOCT2 renal expression in rats (54).

Drug use, in particular trimethoprim, might affect FE_{creat} (14, 21, 22, 33). In the 19 patients on co-trimoxazole in our study, however, we found no effect on FE_{creat}, possibly due to lack of statistical power or the low dose of co-trimoxazole. The type of calcineurin inhibitor (cyclosporin A or tacrolimus), however, affected FE_{creat}, although an older report did find no effect of cyclosporine A on tubular transport of creatinine (19).

In our patients FE_{creat} ranged from 0.71 to 1.39. Of note, FE_{creat} was <1 in 41% of the patients, implicating net tubular creatinine reabsorption. Creatinine reabsorption, albeit infrequent, has been described previously (30, 31, 35, 45). It is considered physiological in newborns and the elderly, and is attributed to tubular characteristics allowing for "back-leak" of creatinine, namely an immature tubular structure with increased permeability in newborns and tubular atrophy in the elderly, respectively (30, 31). Moreover, it has been reported in pathological conditions, such as decompensated heart failure or uncontrolled diabetes (35). In renal transplant recipients, a small study (45) reported several cases with a ratio creatinine clearance/polyfructosan clearance < 1, interpreted by the authors to be due to tubular creatinine reabsorption. Those prior studies were small, however, thus precluding a reliable estimation of the frequency of FE_{creat} <1.

Of note, iothalamate clearance on the average yields values 8 percent higher than polyfructosan (inulin) (3, 4, 36), so our values of FE_{creat} would be correspondingly lower if using different tracer. However, assuming an 8% lower true GFR would still have resulted in an FE_{creat} <1 in 30.8% of our patients (data not shown).

The proportion of patients with FE_{creat} <1 was substantially higher than we found in healthy subjects with the same methodology, where 17% had FE_{creat} <1 (46). Our study was not designed to unravel mechanisms of such differences, but several explanations are possible. First, other populations with FE_{creat} <1 are characterized by impaired tubular functional integrity, either by tubular immaturity (newborns) or tubular atrophy (elderly). This suggests that tubulo-interstitial damage, as commonly present in transplant recipients (41), can contribute to negative FE_{creat}. Drug use, including co-trimoxazole, calcineurin inhibitors, fluoroquinolones, cimetidine or other, affecting OCT2 function could also be involved (14, 21, 22, 33). Finally, the single kidney state of the transplanted kidney leads to elevation of single nephron GFR. In general, tubular functions adapt in proportion to changes in GFR, in order to maintain glomerulo-tubular balance (6, 17, 18, 44). Whether this also applies to the regulation of OCT2, however, has not been demonstrated. Lagging behind of OCT2 up-regulation in the single kidney state could then account for low FE_{creat}. Further studies would be needed to support this assumption.

In line with the effect on FE_{creat}, bias of the MDRD and CKD-EPI equation was significantly associated with the rs316009 minor allele, with a net difference in bias of approximately 9 ml/min between homozygotes for the ancestral and the minor allele for both equations. No such effect on bias was found for the Cockroft-Gault equation, which is in line with its calibration on creatinine clearance. Thus, genetic variability in tubular creatinine handling can induce a systematic error in assessment of eGFR by creatinine-based equations that can lead to spurious associations with eGFR.

Remarkably, and unexpectedly, the *SLC22A2* SNPs were associated with prevalent ESRD with an increased odds ratio for ESRD for the minor alleles of both SNPs. This is in line with the increasing recognition of the role of tubulo-interstitium in the

pathophysiology of renal disease, and, accordingly, the notion that an integrative measure of both glomerular and tubular function may bear more prognostic relevance than a specific measure for GFR only (42). Considering the substrate polyspecificity of OCT2 (59), reduced tubular secretion of various candidate substances might potentially be involved, including catecholamines, dopamine, prostaglandins, advanced glycation end products, and, possibly, uremic toxins, that could affect the kidney either directly or by effects on blood pressure and volume status and hence promote progressive renal damage (16, 20, 28, 32, 56, 60). Finally, the association with ESRD as indicator for the susceptibility to renal damage is corroborated by recent GWAS data reporting a suggestive association between rs316019 and diabetic nephropathy (43).

We did not observe an association between the donor kidney *SLC22A2* SNPs and GF. Although the SNPs effect was direction-consistent with the case-control analysis of ESRD in native kidney, the results were not statistically significant. It might indicate true absence of an association or lack of power to detect a genetic effect on GF, and warrants further investigation in larger transplant populations.

The strength of our study lies in its specific design and the detailed renal phenotype. However, limitations should be mentioned. Our work was a single-center study in a predominantly Caucasian population, which limits generalizability, and we acknowledge an inherent selection bias within ESRD cases towards patients eligible for transplantation. The results on the detailed renal phenotype were obtained in renal transplant recipients only, and hence cannot be generalized to native kidneys. Finally, our in vivo clinical approach only allows solid conclusions on net creatinine handling, and assumptions on OCT2 rely on inference.

Significance / Implications:

Our study represents a hypothesis-driven in vivo follow-up of prior GWAS and in vitro studies. It introduces FEcreat as a novel renal phenotype in genetic studies. *SLC22A2* genotype is associated with phenotypes of net tubular creatinine secretion, namely FE_{creat} and bias of eGFR. Thus, genetic variability of tubular creatinine handling should be taken into account in GWAS analyses on creatinine-based renal function phenotypes, and in this respect profiling the *SLC22A2* variants can improve eGFR performance. Finally, the association of *SLC22A2* SNPs with ESRD is consistent with a role of tubular integrity in the protection against progressive renal damage, but requires further substantiation.

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Chapter 7

Genome-wide association scan of serum urea in a European population

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Introduction

It is well established that a genetic component plays an important role in the multifactorial etiology of renal disease.[1-3] Heritability of renal function indices (serum creatinine, glomerular filtration rate, and creatinine clearance) was demonstrated by several twin and family studies[4] and genome-wide association studies (GWAS) identified a large number of associated loci.[5-7]

Serum urea (also known as blood urea nitrogen, or BUN), along with creatinine, is the most frequently requested measurement of renal function in the assessment of patients with renal disease. Due to different mechanisms of synthesis and clearance, the two markers are not fully equivalent in estimation of renal function, and in some conditions serum urea is considered to be superior to creatinine.[8-10] Urea is the end product of protein catabolism; it is synthesized mainly in the liver and excreted by the kidney. Its blood level depend on numerous factors including hydration status, metabolic rate, dietary protein intake, medication use, liver, cardiac and renal function. Role of putative genetic factors can be also proposed.

The latter has been substantiated by a recent study which estimated heritability for urea to be 0.44 [95% CI 0.35-0.53].[11] This implies that approximately 44% of the inter-individual variation in blood urea could be explained by additive genetic effects, thus indicating a relatively high contribution of genetic factors. Furthermore, a recent GWAS of serum biochemical traits performed in a sample of about 14,300 Japanese subjects[12] found several loci associated with blood urea nitrogen (BUN). However, no genetic loci for this trait have been detected thus far in European populations.

We thus aimed to identify genetic loci influencing blood urea concentration in European subjects. This will help to explain variability in urea levels that exists in the general population and ultimately will provide insight into pathways and physiological mechanisms involved in regulation of this compound.

Methods & Materials

Study population

We performed this study in the population of NESDA (Netherlands Study of Depression and Anxiety) which is an ongoing longitudinal cohort study to examine the prevalence, long-term course and consequences of depressive and anxiety disorders in the adult population. A detailed description of the study design can be found elsewhere.[13]

Briefly, a total of 2981 participants, consisting of a healthy control group, people with a history of depressive or anxiety disorder and people with current depressive and/or anxiety disorder, were recruited from community (19%), primary care (54%) and outpatient psychiatric clinics (27%), and included at the baseline assessment in 2004-2007. Inclusion criteria were a lifetime diagnosis of major depressive disorder

or anxiety disorder, age between 18 and 65 years and self-reported western European ancestry. Persons who were not fluent in Dutch and those with a primary diagnosis of a psychotic disorder, obsessive compulsive disorder, bipolar disorder, or severe substance use or dependence were excluded. Biological sample collection and biobanking procedures, i.e. blood sampling and DNA isolation, took place during the baseline visit and has been previously described in detail.[14]

Imputed data of the Affymetrix 600K genome-wide genotyping array including 2,473,986 post-quality control autosomal SNPs were available for 1925 subjects.

Urea measurement

Urea concentration (mmol/L) was measured in Li-heparin plasma using a kinetic UV enzymatic assay for the quantitative determination of urea on a Roche automated clinical chemistry analyzer.

This method is characterized by high analytical sensitivity (lower detection limit is 0.53 mmol/L) precision and reproducibility (coefficients of variation: within-run 0.8%, between-run 3.45).

Statistical analysis

Urea plasma concentration values were obtained for 1901 subjects with genome-wide SNP genotyping data. The values which exceeded 4 standard deviations from the mean (n=5) were considered to be outliers and were excluded from the subsequent analyses. The final sample thus consisted of 1896 individuals (613 males, 1283 females).

Prior to running genetic association analyses, the phenotype of interest was first explored using PASW Statistics 18.0 (SPSS Inc., Chicago, IL) to test the distribution and correlations with potential covariates. Subsequently, SNPTEST v2.2.0[18,19] was used for genome-wide association testing. The linear regression on single SNP genotypes was run under an additive genetic model, using thresholded genotypes (calling threshold 0.9) and non-transformed, non-standardized urea concentration as a dependent variable. As covariates, sex, age, age^2, body mass index (BMI) and the top ten principal component analysis scores (PCA1-10) were incorporated. Also, to adjust for potential confounding by renal function, serum creatinine or glomerular filtration rate ($eGFR_{creat}$) estimated from

it by the Modification of Diet in Renal Disease (MDRD) Study equation[15] were included as covariates (the values were natural log transformed to approximate normality of the distribution). Additionally, to test for plausible gender-specific associations, we divided the whole sample into males and females and performed the analysis in the two subgroups separately.

We thus ran 9 models depending on covariates used:
1) age, age2, sex, BMI, PCA1-10;
2) age, age2, sex, BMI, PCA1-10, serum creatinine;
3) age, age2, sex, BMI, PCA1-10, eGFRcreat;
4-9) models 1-3 in a sex-stratified manner.

For visualization of the results, WGAViewer v1.26l. 2010[16] was used to build the quantile-quantile (Q-Q) and Manhattan plots.

Results

The Q-Q plot of the total sample consisting of 1896 individuals (**Figure 1**) showed no deviation from the expected distribution, thus, there were no systematic biases causing statistics inflation. Accordingly, the genomic control λ (lambda) was 0.9986, confirming that there were no residual population stratification effects after adjustment for the PCA scores.

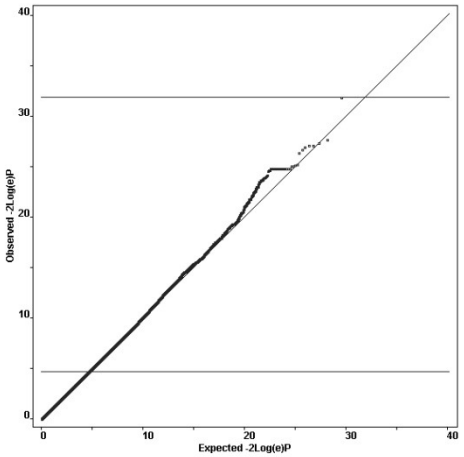

Figure 1. Quantile-quantile plot of the observed vs expected -2 $\log_e$ p values. The lower line indicates the 90th percentile, while the upper one denotes the point where the p values lift off from the expected distribution.

The corresponding Manhattan plot (**Figure 2**), however, showed that none of the SNPs reached the genome-wide significance level of 5×10^{-8} for association with blood urea.

The additional adjustment for serum creatinine or eGFR, as well as running the analyses in a sex-stratified manner, did not materially change the association results or significance magnitude (data not shown).

The three SNPs, identified in the GWAS of BUN in a Japanese population[12], were not significantly associated with the phenotype of interest in our study in European subjects (**Table 1**).

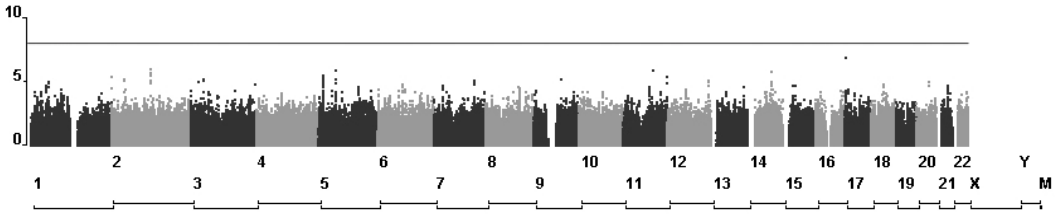

Figure 2. Manhattan plot of -log$_{10}$ p values (y axis) for association with plasma urea by chromosomal location (x axis). The horizontal line denotes the threshold of genome-wide statistical significance (5×10^{-8}).

Table 1. A comparison of the genome-wide association results for blood urea between the previous study in a Japanese population and our present report.

	GWAS in a Japanese population[12]			**Our GWAS** *		
SNPs	*MAF*	*Beta (SE)*	*p*	*MAF*	*Beta (SE)*	*p*
rs9820070	0.31	-0.087 (0.013)	1.24×10^{-11}	0.40	-0.051 (0.036)	0.150
rs11709625	0.17	0.102 (0.016)	1.46×10^{-10}	0.19	-0.020 (0.045)	0.653
rs4890568	0.24	-0.087 (0.014)	2.02×10^{-10}	0.06	-0.126 (0.083)	0.132

* The results of the model with additional adjustment for serum creatinine are presented to allow for comparison with the cited study[12]. MAF, minor allele frequency; SE, standard error of the mean.

Discussion

We present genome-wide association results for plasma urea concentration in European subjects. To our knowledge, this was the first GWAS focusing on this trait in a European population. However, our analyses identified no significant loci for this phenotype. The lack of statistical significance in our study might be explained by the study design issues, namely insufficient sample size and crude phenotype.

We used a relatively small discovery cohort of 1,900 individuals in our GWAS. To gain adequate statistical power to be able to detect SNP effects at genome-wide significance level ($p=5\times10^{-8}$), we would need a substantially larger sample size. To achieve that, GWAS in additional cohorts followed by a meta-GWAS analysis are needed.

However, a recently reported GWAS of serum metabolite levels,[11] although it used a relatively large population of 8,300 individuals, identified no significant loci for blood urea. This might point to the fact that this phenotype presents an analytical challenge.

Plasma urea is a complex and composite phenotype. The levels of urea present in blood are determined by the interplay of numerous factors including (but not limiting to) hydration status, metabolic rate, dietary protein intake, medication use, liver, cardiac and renal function, which might obscure subtle genetic effects. In our study we attempted to account for an influence of kidney function on blood urea by adjusting the association analyses for either serum creatinine or eGFR. Also in the previous GWAS of BUN in a Japanese population adjustment for creatinine was used.[12] However, we were not able to control for potential confounding by other factors. In particular, it would be highly relevant to obtain the information on dietary protein intake and/or urea urinary excretion. Therefore, further studies in more thoroughly phenotyped populations might be useful to dissect the environmental determinants of blood urea and reveal the putative genetic ones.

Given the clear gender dimorphism of many metabolic traits, in particular, blood urea, and the recent evidence of gender differences of genetic variants in metabolism-related genes,[17] we re-analysed the data looking for sex-specific effects, i.e. those restricted to males or females only. However, these gender-stratified analyses revealed no significantly-associated loci of interest. Again, the lack of statistical significance could be attributed to insufficient power due to small sample size.

The three SNPs, identified to be associated with BUN in a Japanese population,[12] were not significantly associated with the phenotype of interest in our GWAS of European subjects. This might be explained by either low statistical power of our study or existence of ethnicity-specific loci for blood urea. The latter is supported by the observation that the frequency of one of the top SNPs, rs4890568, appeared to be substantially different between the populations. In the future, we intend to expand our study by performing GWAS in additional cohorts followed by a meta-GWAS analysis. If this results in detection of significant hits, we will approach the Japanese group for replication of our

findings, which will enable us to discriminate between plausible population-specific and general loci for serum urea.

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Chapter 8

General Discussion

Genetics of common kidney disease: from GWAS to clinical nephrology

Since the first successful GWAS in 2005,[1] major advances have been made by this method to unravel the genetics of common disorders, including kidney disease. Multiple genetic loci have been identified in association with kidney function, CKD, albuminuria and some other renal traits.

Some of the genomic loci, detected through GWAS for common variants, contain also rare mutations of large effect which were already known to cause monogenic diseases of the kidney. For example, the *CUBN* gene, highlighted in the GWAS of albuminuria that identified a common associated polymorphism,[2] harbors mutations causing a rare autosomal-recessive disorder, Imerslund-Gräsbeck disease. Another example is *UMOD*, deleterious mutations in which result in autosomal-dominant renal disorders, namely medullary cystic kidney disease-2 and familial juvenile hyperuricemic nephropathy, while GWAS found common variation in this locus to be associated with renal function traits and CKD.[3-7]

Such a "re-discovery" of Mendelian genes, confirming prior genetic concepts, fuelled confidence in the GWAS approach. Perhaps even more important, GWAS identified genomic regions previously unsuspected of being involved in kidney (patho)physiology, thus providing interesting clues that could lead to discovery of novel pathways of disease, or better understanding of alleged pathways of disease.

However, albeit a powerful approach for gene discovery, GWAS by itself does not provide mechanistic insights. In fact, GWAS generate many interesting hypotheses to be tested in post-GWAS follow-up research. The studies described in this thesis provide such post-GWAS efforts, aimed at carrying the genotype-phenotype associations observed in prior GWASs on renal traits towards the realm of clinical nephrology. To this purpose we selected three loci identified by GWASs for association with eGFR and albuminuria, respectively, based on biological knowledge of their possible role in the kidney, and tested these in a hypothesis-driven approach in smaller association studies where more detail on the renal phenotypes was available.

Functional follow-up of GWAS

In the past few years, a number of genetic loci has been robustly associated with kidney disease. However, statistical association does not equal causality. Absence of proof that the associated gene is also the causal one presents a major bottleneck in the biological translation of GWAS findings.

Multiple lines of evidence are needed to constitute proof of a causal genetic variant, warranting post-GWAS studies on different levels – including bioinformatics (*in silico*), *in vitro* and *in vivo* research.

Bioinformatics prediction of molecular function

Bioinformatics-based SNP prioritization is often the first step of post-GWAS analysis, aimed at predicting the likelihood that variation in a particular SNP has functional consequences. Polymorphisms in coding regions, causing amino acid changes, especially if localized in a conserved region or functional domain of a protein, are likely to have functional consequences. Likewise, SNPs mapped to regulatory regions of a gene, e.g., promoters or splice sites, can be expected to bear functional potential.[8] For example, in **chapter 6** we used bioinformatics to predict consequences of a genetic polymorphism in the *CUBN* gene for the coded protein, cubilin. Bioinformatics thus provides a useful tool to prioritizing SNPs for possible functionality, but subsequent *in vitro* and *in vivo* studies are needed to prove a biological impact of the polymorphisms on the protein level.

Animal models and in vitro studies: functional proof

As genetic manipulations are obviously impossible in humans, cross-species modeling can be used to recapitulate human phenotypes and, thus, validate the genetic discovery in an independent setting. Progress in gene orthology knowledge has made it possible to take advantage of distant animal species in the targeted study of genes that have clinical relevance for human diseases. In mouse, rat, zebrafish and other model organisms genetic engineering can thus be used to mimic the human disease by gene knock out (knock in, knock down) and obtain useful insights into gene function.[9,10] Additionally, *in vitro* studies help to define the molecular mechanisms by which genetic variants affect pathways of cellular or tissue physiology.

In vivo clinical studies

The final proof that a particular genetic variation bears clinical relevance will by definition require clinical studies. Moreover, clinical pathophysiological studies are indispensable in elucidating the genotype-phenotype relationships in terms of underlying mechanisms. Clinically oriented post-GWAS studies, aimed at elucidating genotype phenotype relationships of loci identified in GWAS to be associated with renal traits, constitute the backbone of this thesis. For each of these loci we applied a hypothesis-driven approach, aiming to integrate previous GWASs results with renal phenotypes.

Dissecting renal phenotypes

UMOD

UMOD holds a prominent place among renal loci due to the unique feature that the gene expression product, uromodulin, is excreted into urine and is thus easily available for quantitative assessment.[11-13] Prior studies reported that uromodulin level in the urine is genetically determined: a region upstream from the *UMOD* gene contains rs12917707 and several other SNPs (e.g. rs4293393 and rs13333226) in high linkage disequilibrium (LD) which were repeatedly shown to be associated with urinary uromodulin concentration.[5,14,15] Urinary uromodulin thus constitutes a non-invasive intermediate phenotype that is potentially useful to unravel genotype-phenotype relationships. Chapters 2 through 4 of this thesis, therefore, were devoted to *UMOD* and its possible role in the susceptibility to renal damage.

Urinary uromodulin has been reported to have prognostic value for the subsequent course of renal function in native kidneys. Genetically elevated urinary uromodulin concentration was associated with prevalent and incident CKD.[5] A recent study, moreover, suggested an association between the *UMOD* variant genotype and the risk for ESRD.[7] However, the level of statistical significance was only nominal warranting further investigation. In **chapter 4** we demonstrated that *UMOD* is indeed a susceptibility gene for ESRD, with the minor rs12917707 allele being associated with a lower risk. Such an independent replication of the results, confirming robustness of the previous findings, is of importance for genetic association studies, and warrants further studies on the role of *UMOD* in the susceptibility to renal damage.

Few data are available on uromodulin excretion in renal transplantation, and data on its prognostic value in renal transplantation are lacking altogether. In **chapter 2,** therefore, we investigated urinary uromodulin levels in a large cohort of renal transplant recipients (RTRs) and found excretion of uromodulin to be associated with renal allograft function, morphology and outcome in a peculiar bimodal fashion: both lower and higher uromodulin levels were associated with higher creatinine clearance, lower interstitial fibrosis/tubular atrophy score and reduced risk of graft failure. The bimodal association with renal damage parameters and outcome is at variance with prior data in native kidneys and indicates that urinary uromodulin cannot be considered a straightforward biomarker for renal damage, at least in the setting of renal transplantation. Interestingly, total urinary uromodulin excretion was substantially elevated in RTRs as compared to CKD patients and healthy subjects, which is remarkable considering that RTRs have only one functioning kidney. These data prompted further exploration of the genetic and environmental determinants of urinary uromodulin excretion.

As *UMOD* is expressed exclusively in the kidney, we assumed that kidney (i.e donor) genotype would be associated with urinary uromodulin rather than recipient genotype. In line with our hypothesis, in **chapter 4** we found that urinary uromodulin levels in renal transplant recipients were indeed associated with donor and not recipient *UMOD*

rs12917707 genotype. Thus, the genotype-phenotype association for this intermediate phenotype that was previously reported in the native kidney was now reproduced in the transplanted kidney, with a similar direction of effect. This implies that it is indeed the *UMOD* genotype of the kidney that associates with uromodulin production.

In **chapter 3** we investigated factors influencing urinary uromodulin in healthy subjects and in renal patients. In *part 1* we found that urinary uromodulin concentration exhibits no diurnal or infradian rhythms in healthy individuals. Accordingly, fluctuations in uromodulin excretion over the day closely followed urinary volume, or urinary flow. This finding might be relevant to the high uromodulin excretion in renal transplant recipients, where urine flow per kidney is twice as high as in subjects with two functional kidneys, but this assumption would require further substantiation. Furthermore, we assessed the variability of uromodulin that has useful practical implications for designing subsequent studies. In *part 2* we observed an effect of dietary sodium intake on urinary uromodulin levels in CKD patients, with a trend for decreased uromodulin excretion during low sodium as compared to high sodium diet. Such a direction of the effect is in line with the previous studies in healthy subjects[15,16] and the general population.[17] Our data support the preservation of the response of urinary uromodulin to sodium load in CKD patients. This is of interest, as it might represent a modality to manipulate urinary uromodulin. If the association between higher urinary uromodulin levels and worse renal prognosis would be based on an adverse pathophysiological effect of higher urinary uromodulin levels, such modulation of uromodulin could be of potential benefit. However, our data on the bimodal association with renal damage in renal transplant recipients warrant for cautious interpretation of the implications of urinary uromodulin.

Taken together, our studies provide independent confirmation of *UMOD* as a gene associated with ESRD since we found a minor allele of its variant rs1297707 to be associated with lower risk of ESRD. Furthermore, we showed that it was indeed the kidney *UMOD* genotype that determines urinary uromodulin levels. Finally, our data illustrated that this intermediate phenotype is subject to complex regulation, with not only genetic but also environmental factors influencing its levels. These data support the relevance of *UMOD* for CKD, and warrant prioritization of *UMOD* for further pathophysiological studies, as the mechanisms relating *UMOD*, urinary uromodulin, and the pathogenesis of renal damage are still enigmatic. It is noteworthy here, that, among the substantial number of loci identified for renal traits, and the even larger number of loci for blood pressure, *UMOD* is among the very few that associates with both blood pressure and renal function. Considering the impact of sodium status on urinary uromodulin and the dependency of genotype-phenotype associations on sodium intake,[15] exploration of gene-environment interaction between *UMOD* and sodium status on pathways of renal damage or protection would be a logical target for further study.

CUBN

Prior data on Mendelian disorders, as well as GWAS findings on *CUBN* suggested a potential role for *CUBN* as a renal damage gene. Mendelian mutations in the *CUBN* gene result in a rare autosomal-recessive disorder, namely Imerslund-Gräsbeck disease (OMIM #261100, Finnish type). It typically manifests with megaloblastic B_{12} vitamin-deficient anemia and peripheral neuropathy. The renal phenotype is characterized by mild to moderate proteinuria, that is due to a molecular defect in the cubilin protein, that leads to inefficient reabsorption of low molecular weight proteins in the proximal tubule.[18-20] Of note, a recent GWAS[2] found common variation in the *CUBN* gene to be associated with albuminuria traits in the general population.

According to its pathogenesis, proteinuria associated with cubilin dysfunction can be classified as tubular proteinuria and accordingly has been considered “innocent”, i.e. not leading to renal damage. However, albuminuria is a well-known risk factor for progressive renal function loss, and it would be important to establish or refute the presumed innocence of *CUBN*-associated urinary protein loss.

We, therefore, investigated the association between genetic variation in *CUBN* and the renal susceptibility to damage in **chapter 5**. First, we found that a common variant in the *CUBN* locus was associated with an approximately 40% increased risk for ESRD. Furthermore, a direction-consistent trend was observed for an association between the donor kidney *CUBN* SNP and development of graft failure in renal transplant recipients. Moreover, in the recipients the donor *CUBN* SNP was associated with higher proteinuria levels. Of note, the *CUBN* gene is expressed mostly in the kidney and intestine, and to smaller extent in some other tissues and organs.[21] Our data, demonstrating an association of donor rather than recipient *CUBN* genotype with development of graft failure and proteinuria after transplantation, support involvement of local, intra-renal pathways in processes of transplanted kidney function and survival, rather than systemic influences.

Thus, *CUBN* was associated with phenotypes of progressive renal function loss and proteinuria, and can be considered a renal susceptibility gene, rather than a locus for “innocent” albuminuria. The fact that the *CUBN* variant was associated with both ESRD and GF, with the same direction of the effect, suggests involvement in common pathophysiological pathways for renal function loss that occur in both native and transplanted kidneys.

SLC22A2

A substantial number of genetic loci was associated with serum creatinine and/or $eGFR_{creat}$ as markers of renal function in recent GWASs. However, the association with creatinine-based estimates might not only relate to kidney function in terms of GFR but also to creatinine biosynthesis or its tubular secretion.

In **chapter 6** we followed up a plausible creatinine secretion locus, *SLC22A2*. It encodes the organic cation transporter 2, OCT2 which is a predominant transporter involved in the renal secretion of creatinine. In the kidney it is expressed in the basolateral membrane of proximal tubular cells, where it mediates tubular uptake of creatinine from the peritubular capillaries as an initial step in its vectorial transport to the apical cell membrane followed by secretion into the lumen.

We studied the association of *SLC22A2* polymorphisms with two phenotypes of net tubular creatinine secretion: namely fractional creatinine excretion (FE_{creat}) and bias of estimated glomerular filtration rate (eGFR) in renal transplant recipients. As the *SLC22A2* gene expression is known to be restricted to the kidney, with only scarce extra-renal expression sites,[21] we could avoid unnecessary multiple testing and test only donor kidney genotype for association with recipient phenotypes. In line with our hypothesis, the *SLC22A2* common variant in the donor kidney genotype was associated with FE_{creat}. Accordingly, the variant was associated with eGFR, that is, renal function estimated from creatinine by either the MDRD or the CKD-EPI equation, but not with GFR measured by iothalamate clearance as a gold standard. Thus, the association with eGFR is due to the association with the tubular component of renal creatinine excretion. In line, the *SLC22A2* variant was associated with bias of eGFR, but not with bias of the Cockroft-Gault equation. The latter is modeled on creatinine clearance, and accordingly includes the tubular component of renal creatinine excretion as well.

Remarkably, the *SLC22A2* variants were associated with increased risk of ESRD. This may seem counterintuitive, as the association with FE_{creat} points towards a predominant tubular effect, rather than an effect on renal glomerular function or renal disease. However, the finding is in agreement with the recent recognition of the tubular compartment as an important player in pathophysiology of renal disease and the emerging evidence that an integrative measure of both glomerular and tubular function may bear more prognostic relevance than a specific measure for GFR only. The substrate polyspecificity of OCT2 may be relevant here, as OCT2 is not only involved in the secretion of creatinine, but also in the tubular excretion of biologically active compounds that play a role involved in the pathophysiology of progressive renal damage, such as catecholamines, AGEs, and, allegedly, some uremic toxins.

The observed relationship of the *SLC22A2* variants with ESRD did not translate into association with renal function loss in the transplantation setting, i.e. GF. It is suggestive of differential involvement of this gene in the pathophysiology of native and transplanted kidneys.

Thus, our results demonstrate that the *SLC22A2* gene is not related to glomerular filtration rate, but to creatinine tubular secretion. We introduced FE_{creat} as a novel renal phenotype for genetic association studies. Remarkably, the *SLC22A2* variants were also associated with increased risk of ESRD, thus highlighting the role of tubular function in progressive renal function loss.

Blood urea as a novel renal phenotype

Blood urea, along with creatinine, is the most frequently used measurement of renal function in the assessment of patients with renal disease. It provides an index of the metabolic impact of reduced renal function, specifically reflecting the renal excretion of breakdown products of protein metabolism. As such, it is a phenotype of interest. However, it remains a largely underexplored phenotype, with no associated genetic loci identified in Caucasians thus far. At the same time, heritability studies showed a relatively high contribution of genetic factors to the inter-individual variation in blood urea, in spite of its well-acknowledged environmental component.

We therefore performed a GWAS of this trait in European subjects (**chapter 7**). However, a relatively small size of the discovery cohort so far did not allow for sufficient statistical power to reveal significant loci for this phenotype. Although we detected no significant hits, our study provided an initial step in exploring this renal trait. Subsequent research efforts are warranted to identify specific responsible genetic loci influencing blood urea concentration in European individuals.

Clinical utility of nephrogenetic findings

Prediction of kidney disease risk

The potential to enable a personalized prediction of disease risk has been suggested as one of the translational applications of GWAS.[22-25]

In chapters 4 through 6 we tested variants in plausible genes for association with prevalent ESRD and incident GF. We found that common variants in the *CUBN* (**chapter 5**) and *SLC22A2* (**chapter 6**) loci were associated with increased risk for ESRD (odds ratio (OR) ranging from 1.2 to 1.4), while the *UMOD* SNP (**chapter 4**), on the contrary, conferred protection against ESRD (OR 0.9). Besides, the *CUBN* variant predicted (hazard ratio 1.5) incidence of graft failure in our longitudinal study of renal transplant recipients.

Such a small magnitude of the effects, in agreement with general observations from previous GWAS, is typical for the majority of common variants. As the small ORs are not

useful for predicting risk to individuals, this led to the realization that the main yield of GWAS will probably not be the prediction of individual risk, but rather discovery of biological pathways underlying polygenic diseases and traits.[22-26]

Test performance

In **chapter 6** we showed that genetic variation in the *SLC22A2* locus has consequences for the precision and accuracy of creatinine-based GFR estimation methods. In our study, bias of the MDRD and CKD-EPI equations, but not the Cockroft-Gault equation, was significantly associated with the rs316009 *SLC22A2* SNP. This shows that genetically determined variability in tubular creatinine handling can induce a systematic error in assessment of eGFR by creatinine-based equations. This should be taken into account in GWASs of creatinine-based renal function phenotypes. In this respect, profiling the *SLC22A2* variants and using them as covariates in genome-wide regression analyses of creatinine-based estimates can be suggested to adjust for the genetically determined differences in tubular secretion of creatinine. Yet, quantitatively the effect was small, and of limited clinical relevance.

Future research perspectives

In the general introduction of this thesis we mentioned that all renal function loci, identified thus far by multiple GWASs, collectively account for only 1.4% of the variation in $eGFR_{creat}$. At the same time, heritability of GFR has been estimated to range from 33 to 75%, indicating that between approximately 33 and 75% of the inter-individual variation in GFR can be explained by additive genetic effects.[22,23,27,28] Similarly, although heritability estimates of albuminuria range from 16 to 49%, the top hit of the GWAS of albuminuria traits (urinary albumin-to-creatinine ratio and microalbuminuria) explained only 0.2% of the total variance of this phenotype[22,23]. Such discrepancy between the estimated contribution of genetic factors to disease and the proportion of the phenotypic variation explained by GWAS-identified SNPs is called “missing heritability”.

Some researchers argue that the missing heritability problem is caused by statistical issues, resulting, on the one hand, in overestimation of heritability in twin studies, and, on the other hand, under-detection of associated variants in GWAS due to inadequate sample size and overly stringent significance requirements. The prevailing opinion, however, is that sources of missing heritability include unidentified rare and structural genetic variants, epigenetic changes such as miRNAs, methylation or histone modification, as well as gene × gene and gene × environment interactions.[29,30]

The missing heritability problem thus constitutes a rationale for further research to uncover additional genetic loci in order to identify the "remainder" of the total heritable contribution to common diseases and traits.

To identify previously undetected variants with smaller effect sizes through GWAS, enlarging the sample size of the initial discovery stage is a strategy that is helpful, by increasing the statistical power. In fact, genetic discovery is nowadays usually conducted as a meta-analysis of results from many individual GWASs, with total sample sizes reaching 100,000 individuals and higher. Therefore, collective efforts by consortia of investigators, efficiently joining resources of the international research community, are needed to make the most of the GWAS method. However, while power issues are becoming less of a concern in the current era of the "mega consortium", the progressively smaller effects sizes in follow-up studies indicate that simply enlarging power will not provide the final solution here.

In some of the studies described in this thesis we faced power issues as well. For example, graft failure analyses were probably underpowered to reveal statistically significant genetic effects (**chapters 4** and **6**) or reach convincing statistical significance level (**chapter 5**). Studies in larger renal transplant populations are warranted to reliably demonstrate the presence or absence of an association between the SNPs and incidence of graft failure. Also in our GWAS of blood urea (**chapter 7**) insufficient sample size was a likely cause for the lack of statistical significance. To achieve adequate statistical power for this study, we intend to perform GWASs in additional cohorts followed by a meta-GWAS analysis.

To reveal the putative "hidden" genetic variants still uncaptured by GWASs, fine-mapping and targeted deep resequencing of the previously associated candidate regions provide useful strategies. Contemporary DNA-sequencing technologies using massively parallel pyrosequencing platforms, known as next-generation (NextGen) sequencing, make possible whole exome or even whole genome high-resolution coverage.[31-34] Besides, they enable detection of some structural variants (copy number variants, CNVs),[35,36] thus expanding the range of captured polymorphisms. Such sequencing efforts are expected to result in discovery of a large number of novel, potentially causal, variants.

Another strategy that might aid in discovery of new susceptibility loci is improvement and refinement of phenotyping, by expanding to additional or more accurate measures of renal function that more closely relate to renal (patho-) physiology than simple integrative measures such as eGFR and albuminuria. In this thesis we showed that this strategy can be informative, as illustrated by the novel renal phenotype that we introduced, namely fractional excretion of creatinine (**chapter 6**). Albeit informative, it is not a routinely available measure, so the sample size remained rather limited. Therefore, subsequent genetic association testing in larger appropriately phenotyped cohorts is required to explore this trait further. Furthermore, investigating gene × gene and gene × environment interactions might provide additional novel insights into genetic architecture

of complex, polygenic diseases such as CKD. For instance, in our case-control study of ESRD we found an interaction between two genetic variants in the *CUBN* locus (**chapter 5**). According to our data, the rs7918972 minor allele requires a copy of the rs1801239 minor allele to express its risk phenotype, whereas the minor allele of rs1801239 displays a protective effect in the absence of the rs7918972 minor allele. However, this warrants further experimental functional investigation to determine whether the observed statistical interaction implies also biological interaction.

Furthermore, gene × environment interaction is highly relevant to explore, all the more so because environmental factors are in principle accessible to intervention. Our group previously reported gene × environment interaction for the ACE (I/D) genotype and sodium status[37] and the current data suggest that sodium status interacts with genotype-phenotype associations for *UMOD* as well. Effect of genetic variation in the *UMOD* locus on urinary uromodulin level was reported previously,[5] and independently replicated in our study in renal transplant recipients (**chapter 4**). Dietary sodium affected urinary uromodulin excretion in both healthy individuals[15,16] and CKD patients (**chapter 3**). Whether the genetic effect of UMOD on the susceptibility to renal damage also modulated by exposure to a sodium load is unknown, and interventional studies involving an environmental challenge of high/low sodium diet are needed to elucidate the putative gene × environment (more precisely, gene × diet) interaction here.

Another type of gene × environment interaction with potential clinical relevance can be gene × drug interaction. Pharmacogenetics is an emerging discipline, addressing genetic variation in pharmacokinetics as well as pharmacodynamics, that holds the promise of pharmacotherapy that is better suited to the individual physiological needs than a one-size-fits-all approach.[38-41] Our studies did not specifically address pharmacogenetic questions. However, it is interesting to note that several drugs are known to affect tubular secretion of creatinine.[42-45] In our study we found also the *SLC22A2* genetic variant to be one of the determinants of creatinine secretion (**chapter 6**). It would be highly interesting to investigate whether there is an interaction between the drug and gene effects and what the modality of such interaction is. Moreover, the association of *SLC22A2* and risk for ESRD prompts to investigate the long term renal risks of drugs affecting creatinine secretion, such as co-trimoxazole.

A recently proposed promising approach is to integrate gene association analyses with the functional biological knowledge on gene expression (transcriptomics, proteomics, metabolomics) and its regulation (regulomics, epigenetics, microRNA profiling) to reveal molecular mechanisms underlying the genetically determined phenotypic variance.[46]

As a high degree of cellular heterogeneity of the kidney and concomitant complexity of its physiology pose a challenge for understanding and interpreting straightforward genotype-phenotype associations, integration of all the biological data at a systems level seems only appropriate to gain an insight into complex renal traits[47-51] (**Figure 1**). We believe that integrating data on DNA variation, transcription and phenotype has the potential to enhance identification of the associations between DNA variation and

complex diseases like CKD, as well as characterize those parts of the molecular networks that drive the disease. As contemporary research goes beyond a single-gene focus, integrative approaches, aimed at analyzing high-dimensional biological data by merging data from multiple sources, will significantly enhance the identification of key drivers of complex disease beyond what could be achieved by genetic association studies alone.

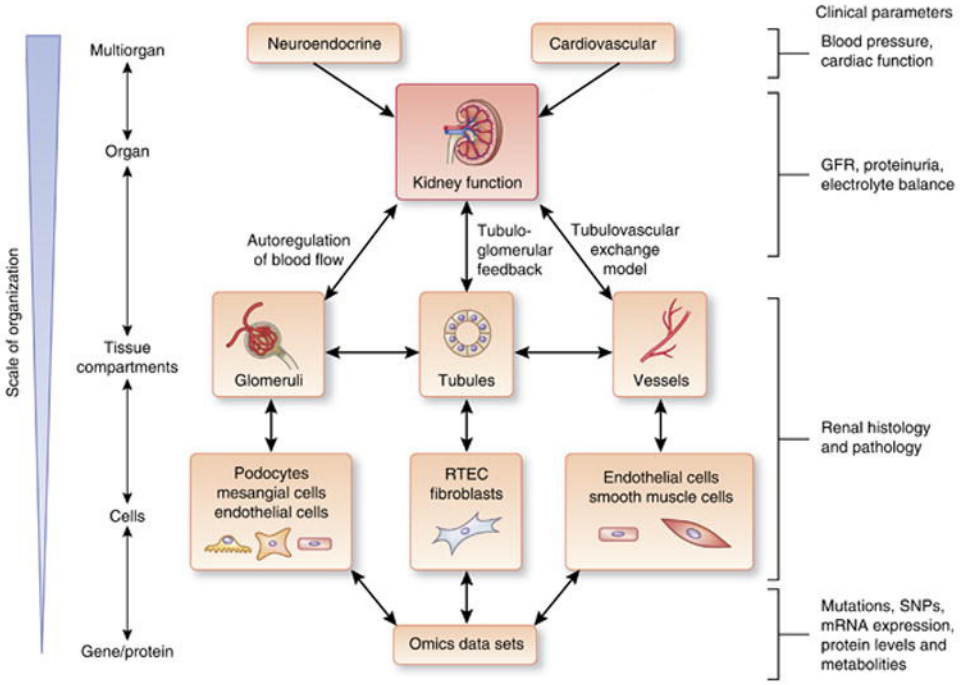

Figure 1. Multiscale analysis of kidney function (adapted from *He et al Kidney Int 2012*)

Conclusions

In the contemporary era of intensive genetic research and rapid generation of large scale data on genotype-phenotype associations, our results illustrate the relevance of dedicated clinical studies for the translation of genotype-phenotype associations from GWAS to the setting of clinical nephrology. Our data illustrate that refinement and dissection of the renal phenotype, as with FE_{creat} and true GFR instead of eGFR, allow better insight into the functional and clinical consequences of the genetic variant. Also, they show the potential of genetics as a dissection tool: our data on the association of *SLC22A2* with ESRD strongly suggest that functional tubular changes may contribute to further renal function loss. This would have been notoriously difficult to show in a merely phenotypic study, where it is almost impossible to dissect cause and consequence in the association between tubular functional changes and subsequent ESRD.

Our data also illustrate that the translation of GWAS data on renal traits to clinical nephrology is neither simple not straightforward. Yet, by integrating the newly generated data with knowledge from clinical pathophysiology, new insights are obtained that eventually contribute to elucidation of the genetic basis of susceptibility and progression of CKD. This may ultimately support the development of novel tools for diagnosis, prevention, prediction and treatment, to translate, all in all, into benefit for the renal patient.

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Nederlandse Samenvatting

In de Westerse wereld heeft ongeveer 10% van de volwassen bevolking een vorm van chronische nierschade, en dit aandeel zal naar verwachting verder toenemen, ten gevolge van de toenemende levensverwachting en de toename van overgewicht/obesitas en type 2 diabetes mellitus. Doordat nierziekten gepaard gaan met een sterk verhoogd risico op hart- en vaatziekten is de morbiditeit en mortaliteit onder nierpatiënten hoog. Tevens is de kwaliteit van leven van de nierpatiënt duidelijk verminderd, met name indien nierfunctievervangende therapie in de vorm van dialyse (nierspoelen) nodig is. Een chronische nierziekte kan vele oorzaken hebben waardoor het ziektebeeld als entiteit een uitgesproken heterogeen karakter heeft. Genetische factoren spelen een belangrijke rol in het ontstaan en ernst van het ziektebeeld en identificatie van deze genetische factoren is belangrijk om kennis over nierziekten te vergroten, maar ook om het diagnosticeren en het behandelen ervan te verbeteren.

In de afgelopen decennia heeft genetisch onderzoek ons veel geleerd over de zogenaamde “Mendeliaanse” nierziekten, een groep nierziekten die het directe gevolg zijn van één enkele DNA-mutatie. Tot deze groep nierziekten behoren bijvoorbeeld de aandoeningen: “autosomaal dominante polycysteuze nierziekten” en “syndroom van Alport”. Bij andere nieraandoeningen, de zogenaamde complexe ziekten, is er niet een overwegende rol van één mutatie, maar zijn er effecten van meerdere genetische varianten, die elk op zichzelf een relatief kleine bijdrage leveren. Daarnaast zijn er ook bijdragende omgevingsvarianten.

Door middel van Genome Wide Association Studies, dat wil zeggen het genotyperen van het gehele genoom, en koppeling van de variatie daarin aan klinische gegevens, is de laatste jaren een snel toenemende hoeveelheid gegevens verkregen over de associatie van bepaalde ziektekarakteristieken, zoals bloeddruk of mate van nierfunctie, met bepaalde variaties in het DNA, ook wel single-nucleotide polymorfismen (SNPs) genoemd. Door middel van GWAS worden nu veel SNP’s ontdekt die mogelijk betrokken zijn bij het ontstaan en progressie van nierziekten. De eerste studie die gebruikmaakte van de GWAS-methode in grote humane cohorten werd in 2005 gepubliceerd. waarna een toenemend aantal ziektekarakteristieken met behulp van deze methode zijn onderzocht. Het aantal neemt inmiddels zeer snel toe, en daarmee ook het aantal met klinische kenmerken geassocieerde SNPs. Een nadeel van de GWAS-methode is dat het alleen associaties blootlegt, maar geen specifieke informatie oplevert over het mechanisme waarop een bepaalde genetische verandering tot een bepaalde ziektekarakteristiek leidt. Daarvoor is dus vervolgonderzoek nodig. Voor SNPs die geassocieerd zijn met variaties in nierfunctie, is het niet alleen belangrijk om onderliggende mechanismen te onderzoeken, maar ook, of de betreffende SNPs ook geassocieerd zijn met een verhoogde kans op klinisch relevante nierschade, in het bijzonder eindstadium nierfalen. In dit proefschrift onderzoeken we voor een aantal SNPs die recent werden geassocieerd met nierfunctie, de relatie met het risico op eindstadium nierfalen, en mogelijke onderliggende mechanismen. Daarmee proberen we de resultaten van GWAS-onderzoek te vertalen naar de klinische praktijk.

Voor dit proefschrift onderzochten we SNPs in een drietal genen waarvan recent met GWAS een verband met nierfunctie is gevonden. Dit zijn: (1) *UMOD*, dat codeert voor het eiwit uromoduline, het meest voorkomende eiwit in urine; (2) *CUBN*, dat codeert voor het eiwit cubilin dat een rol speelt bij de terugresorptie van eiwit in de nier; en (3) *SLC22A2*, dat codeert voor een eiwit dat een rol speelt bij de uitscheiding van afvalstoffen door de nier.

Hoofdstuk 2 t/m 4 richt zich op het eiwit uromoduline, dat wordt uitgescheiden in de urine en daardoor eenvoudig meetbaar is. Eerder werd al aangetoond dat de uitscheiding van uromoduline in de urine het beloop van de nierziekte kan voorspellen, en bovendien dat genetische variatie in het *UMOD* gen mede de uitscheiding van uromoduline in de urine bepaalt. Daarmee zou het effect van genetische variatie in het *UMOD* gen op simpele wijze in de urine meetbaar zijn. Wij onderzochten dit in een aantal studies in patiënten met nierziekte, in patiënten na niertransplantatie, en in gezonde vrijwilligers. In **hoofdstuk 2** onderzochten we of de uitscheiding van uromoduline in de urine het falen van het niertransplantaat kan voorspellen. We vonden dat de uitscheiding van uromoduline bifasisch geassocieerd is met de functie van het niertransplantaat. Zowel een lagere als hogere excretie van uromoduline in de urine was geassocieerd met een betere nierfunctie, minder littekenvorming in de nier en minder verlies van niercellen, waardoor het transplantaat minder schade oploopt. Deze bifasische associatie is niet in overeenstemming met eerder onderzoek in nierpatiënten zonder niertransplantaat (dus in de eigen nieren). Dit lijkt te suggereren dat meten van uromoduline in de urine weinig toegevoegde waarde heeft in niertransplantatiepatiënten; ook geeft het aan dat de relatie tussen uromoduline in urine en nierschade blijkbaar complexer is dan verwacht. In **hoofdstuk 3** onderzochten daarom in meer detail de rol van niet-genetische factoren bij de concentratie uromoduline in de urine, bij gezonde vrijwilligers en nierpatiënten. In het eerste gedeelte van de studie onderzochten we de 24-uursvariatie in de uitscheiding van uromoduline, alsmede de dag-tot-dag variatie bij vrijwilligers. De uitscheiding van uromoduline had bij hen geen duidelijk dag-nacht ritme, en er was ook weinig intra-individuele variatie van dag tot dag. Uromoduline heeft echter wel een duidelijke inter-individuele variatie, hetgeen implicaties kan hebben voor verdere vervolgstudies. In het **tweede gedeelte** van de studie onderzochten we de effecten van zoutbeperking en farmacologische interventie op de uitscheiding van uromoduline bij nierpatiënten. We vonden dat de uitscheiding van uromoduline in de urine lager is tijdens zoutbeperking wat in overeenstemming is met resultaten in gezonde vrijwilligers. In **hoofdstuk 4** vonden we dat *UMOD* een duidelijke relatie heeft met de concentratie uromoduline in de urine van patiënten na niertransplantatie. Bovendien was *UMOD* geassocieerd met het risico op eindstadium nierfalen. Samenvattend ondersteunen onze studies een rol van het *UMOD* gen bij het ontstaan en beloop van nierziekten. De uitingsvorm van dit gen – namelijk de concentratie uromoduline in de urine – wordt op complexe wijze wordt gereguleerd, met een belangrijke bijdrage van niet-genetische factoren. Het mechanisme van de relatie tussen variatie in het *UMOD* gen en nierschade blijft vooralsnog onduidelijk: wel is opmerkelijk dat variatie in het *UMOD* gen ook betrokken is

bij bloeddrukregulatie. Dit zou potentieel van belang kunnen zijn voor de rol bij nierschade.

Het tweede onderzochte gen betreft het *CUBN* gen. Dit codeert voor het eiwit cubiline. Het *CUBN* gen is betrokken is bij de nierziekte “Imerslund-Gräsbeck”, een zeer zeldzame nierziekte die veroorzaakt wordt door mutaties in het *CUBN* gen. Deze ziekte gaat gepaard met eiwitverlies in de urine (proteïnurie) ten gevolge van een inefficiënt verlopende reabsorptie van bepaalde eiwitten in de nier ten gevolge van een niet goed werkend cubiline. Hernieuwde aandacht voor cubiline is ontstaan doordat GWAS-onderzoek aantoonde dat andere, frequenter voorkomende variaties in het *CUBN* gen samenhangen met proteïnurie in de algemene bevolking en bij patiënten met nierschade. Proteïnurie is een bekende risicofactor voor nierfunctieachteruitgang, waarbij meer verlies van eiwit in de urine samenhangt met meer nierschade en een slechtere prognose. In hoeverre deze variaties in het *CUBN* gen daardoor geassocieerd zouden kunnen zijn met het risico op nierschade, was echter niet bekend. Wij testten daarom de hypothese dat variatie in het *CUBN* gen geassocieerd is met het risico op eindstadium nierfalen. In **hoofdstuk 5** hebben we daartoe, in ontvangers van een niertransplantaat, onderzocht of genetische variatie in het *CUBN* gen een relatie heeft met nierschade (in de vorm van proteïnurie) en met progressieve nierfunctieachteruitgang (gedefinieerd als terugkeer naar dialyse of anderszins falen van het niertransplantaat). Ten eerste vonden we dat een veelvoorkomende *CUBN* SNP samenhing met een ongeveer 40% hoger risico op falen van het niertransplantaat. Bovendien was het *CUBN* SNP van de donornier geassocieerd met meer proteïnurie in de niertransplantatiepatiënt. Deze SNP is dus geassocieerd met zowel proteïnurie, als met een verhoogde kans op progressief nierfunctieverlies.

In **hoofdstuk 6** onderzochten we de manifestaties van genetische variatie in het gen voor *SLC22A2*. Eerder werd, in GWAS studies, gevonden dat deze SNP een relatie heeft met de nierfunctie, gemeten met behulp van creatinine. Werd de nierfunctie gemeten met behulp van Cystatine-C, dan was er geen relatie. Dit leidde tot de hypothese dat genetische variatie in *SLC22A2* leidt tot een effect op serum creatinine door een effect op tubulaire secretie, temeer daar *SLC22A2* in de niertubulus een transport functie heeft. We onderzochten daarom de relatie tussen tubulaire uitscheiding van creatinine en variatie in het gen voor *SLC22A2*: hiertussen bestond inderdaad een verband, terwijl er met glomerulaire filtratiesnelheid juist geen verband aanwezig was. Opmerkelijk was, dat er ook een verband werd gevonden tussen genetische variatie in *SLC22A* en het risico op eindstadium nierfalen. Blijkbaar is een goede tubulaire transportfunctie beschermend tegen progressief verlies van nierfunctie.

Tot slot hebben we in **hoofdstuk 7** zelf de GWAS-methode gebruikt om SNP’s te ontdekken die verband houden met de regulatie van ureum in het bloed. Doordat onze patiëntengroep relatief klein was hadden we echter onvoldoende statistische kracht om deze SNP’s te ontdekken. Aanvullend onderzoek is dus nodig om deze SNP’s te kunnen lokaliseren.

Samenvattend illustreert dit proefschrift het belang van nauwkeurig uitgevoerde klinische studies voor de vertaling van de resultaten van GWAS-onderzoek naar de klinische praktijk. Onze data laat zien dat het analyseren van specifieke ziektekarakteristieken ons betere inzichten kan geven in de invloeden van verschillende genetische varianten op de ontwikkeling van nierschade. Deze nieuwe inzichten kunnen op termijn bijdragen aan het ontrafelen van de genetische basis van nierziekten, en daarmee op termijn mogelijk leiden tot betere therapieën voor nierziekten.

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Author's Biography

Curriculum Vitae

The author of this thesis was born on August 15, 1985, in Novomoskovsk, Dnipropetrovsk oblast, Ukraine.

She completed primary and secondary school (1992-2000) in her home town. After she won the 3rd prize in the Regional competition ("Olympiad") in Biology among secondary school students (Dnipropetrovsk, 2000), she was admitted to Dnipropetrovsk Regional Medical Lyceum where she studied from 2000 to 2002. As she then won the 3rd prize in the National Olympiad in Biology among high school students (Lviv, 2002), she subsequently enrolled Dnipropetrovsk State Medical Academy off-competition (i.e. without entrance examinations).

As a medical student, she won a number of prizes in different National competitions among universities undergraduates, such as Medical Biology and Parasitology (2nd prize, Lugansk, 2003), Human Physiology (1st prize, Donetsk, 2005), Medical Molecular Biology, Cytology and Genetics (1st prize, Kiev, 2005), Pathophysiology (3rd prize, Simferopol, 2005) and other.

While studying medicine, she gradually developed ever since growing interest in research. She became a member of students' scientific societies of Medical Biology, Physiology and Urology & Nephrology, and got involved in their research activities, first by joining on-going projects, and, later, designing experiments and initiating her own projects.

In 2006, she presented some of her research results at the 17th European Students' Conference (ESC) in Berlin, Germany, and won a prize for the best oral presentation in the session "Nephrology/Urology". She received the "Award for significant successes in research work" by Academy of Medical Sciences of Ukraine and Association of Medical Schools of Ukraine (2007). In 2007, she came to Groningen, the Netherlands, for the first time, to participate in the 14th International Student Congress of Medical Science (ISCOMS). She gave an oral presentation and then took part in the ISCOMS Research Fellowship at the Department of Nephrology. Subsequently, she graduated from the Medical Academy with the M.D. degree, *summa cum laude* (2008) and came to Groningen to start a PhD project.

From 2008 to 2012 she has been performing her doctoral research described in this thesis at the Department of Nephrology, University Medical Center Groningen, under supervision of Prof. dr. G.J. Navis, Prof. dr. H. Snieder and Dr. M. Seelen.

After her promotion, she is going to pursue her research career as a post-doctoral associate at the Jackson Laboratory (Bar Harbor, ME, USA) where she will continue investigating genetic architecture of kidney disease under supervision of Dr. Ron Korstanje.

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Other qualifications & skills:

A.A. in Music, Major: classical piano

Visual arts: drawing, photography, 3D modeling. Portfolio website: http://www.pbase.com/ar

Sports: Taekwondo, 7th Dan (green belt)